Dizertačná práca

Imunohistochemická analýza proteínových markerov CD9 a CD29 u „triple“ negatívnych karcinómov prsníka

MUDr. Róbert Ondruššek

Školiteľ dizertačnej práce: doc. Mgr. Jan Bouchal, Ph.D.

predchádzajúca školiteľka: doc. MUDr. Světlana Brychtová, Ph.D.

Univerzita Palackého v Olomouci

Ústav klinické a molekulární patologie LF

Študijný obor: Patologická anatomie a soudní lékařství

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Prehlásenie

Prehlasujem, že som svoju dizertačnú prácu

„Imunohistochemická analýza proteínových markerov CD9 a CD29 u „triple“ negativných karcinómov prsníka“

vypracoval samostatne a použil som len textové pramene, ktoré citujem a uvádzam v bibliografii.

MUDr. Ondruššek Róbert

Poďakovanie

Vyslovujem poďakovanie vedúcemu Ústavu molekulárnej a klinickej patológii LF UPOL a môjmu školiteľovi dizertačnej práce doc. Mgr. Janovi Bouchalovi, Ph.D., a doc. MUDr. Svetlane Brychtové Ph.D. a ich všetkým spolupracovníkom za umožnenie vypracovať dizertační prácu na ich ústave a zároveň ďakujem za ich pomoc, venovaný čas, veľa nápadov a cenných pripomienok, ktoré prispeli k zlepšeniu práce. Moja vďaka patrí aj Mgr. Karlovi Součkovi Ph.D. a jeho všetkým kolegom z Laboratoře cytokinetiky Biofyzikálního ústavu AV ČR v Brně za dobrú spoluprácu.

OBSAH

1. Teoretický úvod

1.1. Mamárne karcinómy

Mamárne karcinómy (BC, breast cancer) sú najčastejším malígnym nádorom žien, a tvoria asi 24% všetkých malignít vo svete (Bray a kol., 2018). Je to heterogénna skupina chorôb a presná klasifikácia nádoru do klinicky relevantných podskupín je rozhodujúca pre prognózu a výber liečby. O onkologickej liečbe rozhoduje histologický grade tumoru, status hormonálnych receptorov spolu so stavom ľudského epidermálneho rastového faktora 2 (HER2/ERBB2), proliferačná aktivita nádoru, patologický stav alebo rozsah nádorovej choroby v podobe TNM klasifikácie a vek pacienta. Podľa imunohistochemických parametrov a proliferačnej aktivity, a podľa molekulárnej génovej charakteristiky boli rozdelené karcinómy prsnej žľazy do 5 hlavných podskupín: luminal A, luminal B, „Her 2 enriched“, “basal like“, a „ normal breast like“ skupina (Perou a kol., 2000). Rozdelenie BC podľa charakteristiky 50 génov (PAM50 klasifikácia), neskôr s pridaním dodatočnej skupiny tzv. „claudin -low“ bolo využité pre predikciu patologickej odpovede na neoadjuvantnú onkoterapiu (Sorlie a kol., 2003) a dali základ pre TCGA a METABRIC klasifikácie karcinómov prsnej žľazy. Táto klasifikácia BC (Curtis a kol., 2012) bola so zameraním nádorov na vedúcu hlavnú tzv.“driver“ mutáciu.

Biológia týchto podskupín nádorov odráža ich rozdielnu povahu, odpoveď na liečbu a prognózu. Moderné technológie a metódy na molekulárnej úrovni pomohli charakterizovať genetické zmeny a charakteristiky nádorov s možno cielenou onkologickou terapiou.

1.1.1. Triple negatívne karcinómy prsnej žľazy (TNBC)

Súčasťou BC je zvláštna skupina nádorov-„triple“ negatívne karcinómy prsnej žľazy (TNBC). TNBC je skupina nádorov väčšinou s agresívnym správaním u recidivujúcich tumorov s následným horším dlhodobým prežívaním s nepriaznivou prognózou v porovnaní s non TNBC (Allison a kol., 2019, Bauer a kol., 2007, Blows a kol., 2010, Liedtke a kol., 2008). Tvorí asi 15% všetkých BC, a sú charakterizované výraznou heterogenitou. Imunohistochemické metódy definujú „triple“ negatívny mamárny karcinóm (TNBC) ako karcinóm s negatívnou expresiou estrogenového receptoru (ER), progesteronového receptoru (PR) a receptoru ľudského epidermálneho rastového faktoru 2 (HER2), zároveň s negativitou amplifikácie génu metódou FISH (Bauer a kol., 2007). Biologické chovanie a patologická odpoveď TNBC na liečbu podľa klasifikácie PAM50, ktorá rozdeľovala TNBC na 2 skupiny („basal like“ a „non basal like“) bolo rozdielne, a nepredikovalo typ patologickej odpovede a biologické správanie týchto nádorov (Hu a kol., 2007). Ďalšie klasifikácie BC so zameraním na TNBC vznikli za účelom stratifikácia nádorov s vyšším a nižším rizikom agresívnosti biologického správania ich prediktívnej patologickej odpovede na terapiu a vznik následnej účinnej cielenej terapie.

1.1.1.1.Klasifikácia podľa Lehmana

Klasifikácia podľa Lehmana a spol (Lehman a kol., 2011, Garrido-Castro a kol., 2019) na základe expresie ESR1, PGR a ERBB2 rozdelila TNBC do 7 zvláštnych podskupín: „basal like1“ (BL1), „basal like2“ (BL2), „immunomudulatory“ (IM), „mesenchymal“ (IM), „mesenchymal stem - like“ (MSL), „luminal androgén receptor“ (LAR) a “unstable cluster (UNS typ). Každý podtyp je charakterizovaný odlišnými genetickými zmenami z hľadiska rozdielnej expresie RNA a somatických mutácií a genetických patologických ciest a kľúčových vedúcich mutácií. BL1 typ je bohatý na gény zapájajúce sa do poškodzovania DNA a do regulácií bunkového cyklu (prítomnosť *TP53* mutácie, amplifikácia *MYC*, *CDK6*, *CCNE1*, delécia *BRCA2*, *PTEN*, *MDM2* a *RB1* géne) (Garrido-Castro a kol., 2019). BL2 typ charakterizuje vysoký level rastových faktorov, vysoká metabolická aktivita a proliferačný fenotyp. IM charakterizujú gény zapojené do spracovania a prezentácie antigénu, signalizácie imunitných buniek a cytokínov (JAK/ STAT, TNF, NFkB) (Garrido-Castro a kol., 2019).

M/ MSL typ je bohatý na gény, ktoré ovplyvňujú bunkovú motilitu, bunkovú diferenciáciu, angiogenézu a proces EMT. LAR typ obsahuje gény s elevációu mRNA a vysoký level proteínu AR, obsahujú početné mutácie v génoch *PIK3CA*, *KMT2C* a *CDH1.* Biologické spávanie TNBC, hlavne typu M/MSL, podľa Lehmana spojený s EMT - zaujímavým javom, spojeným s progresiou zhubného nádoru a jeho metastázovania (Kalluri a kol., 2003, Kalluri a kol., 2009).

1.1.1.2. Klasifikácia podľa Bursteina

V tejto klasifikácii (Burstein a kol., 2015) boli prítomné 4 stabilné molekulárne subtypy podľa mRNA profilovania: luminal androgén receptor (LAR), mesenchymal (MES), basal-like immune-suppressed (BLIS), and basal-like immune-activated (BLIA). mRNA profilovanie nádorov dali do vzťahu s imunohistochemickými metódami založenými na expresii markerov: AR, Claudín 3, E cadherín, CK5/6, EGFR a IDO1, a FOXC1. „Basal like“ subtypy TNBC boli stratifikované podľa statusu IDO1 a FOXC1. BLIA typ bol definovaný ako „basal like“ tumor, ktorý bol IDO1 pozitívny a FOXC1 negatívny. BLIS typ definoval imunohistochemicky status IDO1 negatívny a FOXC1 pozitívny.

LAR typ bol asociovaný s vyšším vekom, apokrinnými histologickými obrazmi, nízkou denzitou stromálnych TIL, a nízkym proliferačnou aktivitou Ki 67. MES typ bol spojený s metaplastickými histologickými črtami. „Basal like“ typy boli spojené s mladou vekovou kategóriou, vysokým histologickým grade, vysokou proliferačnou aktivitou Ki67 a vysokou denzitou TIL. BLIS typ mal najhoršiu prognózu z týchto všetkých skupín TNBC (Kim a kol., 2018).

1.1.2. Onkologická liečba TNBC

U pacientov s TNBC neprichádza do úvahy systémová liečba v podobe hormonálnej endokrinnej terapie a tiež cielenej anti HER terapie. Na prvou mieste sa používa chemoterapia v podobe taxánov (docetaxel, paclitaxel), alebo antracyklínov, alebo ich kombinácii. Menej sa používajú iné chemoterapeutická v liečebných schémach cyclophosphamid, metotrexát a flourouracil. Ďalšou liečebnou modalitou sú EGFR inhibítory pri nádoroch s pozitívnou expresiou proteínu EGFR. Ďalšími modalitami sú inhibítory a špecifické protilátky proti rastových vaskulárnym faktorom, ktoré podporujú angiogenézu anti VEGF (bevacizumab a iné).

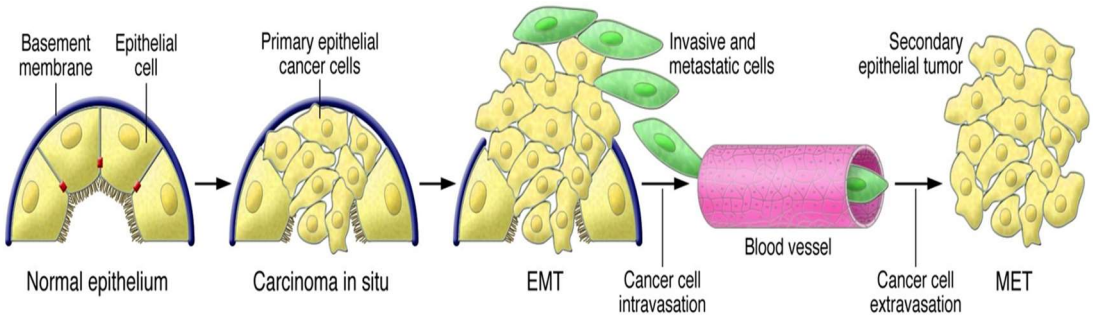
Sľubným liekom u BRA1 a BRCA2 pozitívnych nádorov, ktoré nesú mutáciu génov *BRCA1/2 (asi u 10% TNBC pacientov)* sú inhibítory PARP poly(ADP-ripose) polymeráza, enzýmov zapájajúcich sa do opráv DNA buniek (Garrido-Castro a kol., 2019, Reyes a kol., 2018). PARP inhibítory spôsobujú poruchy mitotickej aktivity buniek so zablokovaním bunkového cyklu a apoptózy nádorových buniek (Garrido-Castro a kol., 2019, Reyes a kol., 2018).

Novou érou terapie je imunoterapia pomocou inhibítorov imúnnych buniek („immune check point“ inhibítory) (Reyes a kol., 2018). U TNBC hlavne typ IM, (dle Lehmana), alebo u nádorov s dôkazom imunitných signatúr bol dokázaný benefit z imunoterapie , ktorý bol dokázaný u cca 20% pacientov s TNBC (Yin a kol., 2018), PDL1 inhibitory sa ukázali ako potenciálne látky, ktoré môžu podstatne zvýšit prežitie a kvalitu života u pacientov s nádoromi prsnej žlazy, hlavne pacientov s TNBC. (Garrico Castro a kol., 2019, Chen kol., 2015). Kombinácia PDL1 inhibítorov a chemoterapie má svoje miesto a je účinná (Garrido-Castro a kol., 2019, Yin a kol., 2018)

1.2. Epitelo-mezenchymálna tranzícia (EMT)

EMT je jav, ktorý umožňuje epitelovej polarizovanej bunke spojenej s bazálnou membránou podstúpiť viaceré biochemické zmeny, ktoré jej umožňujú prijať fenotyp mezenchymálnej bunky. EMT sa vyskytuje v 3 odlišným biologických prostrediach, ktoré majú rôzne funkčné dôsledky.

Z hľadiska našej práce je najdôležitejším EMT 3.typu, ide o EMT spojené s progresiou karcinómov a ich metastázovaním, kedy sa mení fenotyp nádorovej bunky za účelom ľahšieho prenikania a šírenia sa bunky do strómy lokálne, i ďalej do krvi s vytváraním vzdialených metastáz, a tým progresie nádoru. Ide o EMT, ktoré hrá úlohu pri všetkých solídnych zhubných nádoroch v procese metastázovania (Kalluri a kol., 2003, Kalluri a kol., 2009). Reverzný jav ako EMT označuje termín MET (mezenchýmovo-epiteliálna tranzícia) (obr.1).



Obr. 1 Epitelovo-mezenchymálna tranzícia a metastázovanie. Epitelová nádorová bunka stráca epitelový fenotyp stratou priľnavosti bunky k bazálnej membráne, a vzájomne k epitelovým bunkám medzi sebou, a vycestuje do stromy, a potom ďalej do krvných ciev. V cielovej tkáni pak založí kolónie nádorových buniek ako základ novej metastázy nádoru, a v novom makroprostredí prejde do pôvodného stavu bunky s epiteliálnym fenotypom javom opačným - tzv. MET. (zdroj: prevzaté z „Kalluri a kol., 2009“)

EMT 1. typu je EMT, ktoré je spojené s implantáciou a vývojom embrya a vznikom orgánov plodu, kde sa vyskytujú spolu rôzne typy mezenchymálnych buniek. EMT, ktoré je spojené s hojením rán a regeneráciou tkanív pri traume a zápale sa nazýva 2. typom EMT. Tento jav je spojený s poškodzovaním tkanív a zápalom. Keď tieto inzulty a zápal pominie EMT sa končí.

V EMT 3. typu, tzv. mezenchymálny fenotyp zahŕňa zvýšenú migračnú kapacitu, invazívnosť, zvýšenú odolnosť voči apoptóze a výrazne zvýšenú produkciu komponentov extracelulárnej matrix (ECM-tzv. mikroprostredie nádoru) (Kalluri a kol., 2003, Kokkinos a kol., 2007).

Kokkinos a kol. definujú EMT ako stav, keď sa cytosklelet buniek tvorených skeletálnou sieťou bohatou na keratíny, charakterizovanou tesným medzibunkovými spojmi a hemidesmosómami, mení na cytoskeletovú sieť bohatú na vimentín s fokálnymi medzibunkovými spojmi - adhéziami (Kokkinos a kol., 2007).

Proces EMT definuje prítomnosť, alebo zvýšená expresia niektorých mezenchymálnych markerov a absencia, alebo znížená/zvýšená expresia epitelových proteínových markerov ako vimentín, E cadherín a N cadherín (Kalluri a kol., 2009). Vimentín je dôležitý vláknitý proteín, poskytujúci štrukturálnu a funkčnú podporu nádorovej bunke. Udržuje mechanickú integritu nádorových buniek pri EMT (Liu a kol.,2015). V počiatočnej fáze rozvoja nádoru je koncentrácia vimentínu veľmi nízka, ale pri progresii tumoru s vyšším pTNM sa zvyšuje (Usman a kol. ,2021, Patel a kol., 2015). Najznámejšou biochemickou zmenou spojenou s EMT je absencia expresie E cadherínu ako intercelulárneho adhezivného proteínu (Medici a kol., 2008, Gheldof a kol., 2014, van Roy a kol., 2009, Vleminckx a kol., 1991),

ktorý zabezpečuje stabilitu tesných adherentných spojov medzi bunkami a je kľúčovým pre zachovanie epitelového fenotypu buniek a stabilitu tkanív (van Roy a kol., 2008). Počas procesu vzniku malígneho tumoru bolo popísaných niekoľko mechanizmov, pri ktorých dochádza k poruchám funkcie E cadherínu (Roy a kol., 2009, Strumane a kol., 2004, Guilford a kol., 1998, Berx a kol., 1998, Sundfeldt a kol., 2003, Kleer a kol., 2001). N cadherín ako ďalší zástupca cadherínov má opačnú úlohu ako E cadherín, podporuje inváziu a progresiu nádoru, tým, že zvyšuje interakciu nádorových buniek so strómou a endotéliami ciev s vyšším rizikom vaskulárneho šírenia nádoru (Qi a kol., 2005), a tiež mechanizmom zvýšenej motility a produkcie proteáz myofibroblastov nádorovej strómy (Derycke a kol., 2006). EMT napomáha progresii TNBC i pomocou mechanizmu rezistencie nádoru na terapiu (Kvokačková a kol., 2021).

V procese EMT a reverzného deja označeného ako MET sa veľmi skloňuje tzv. plasticita u nádorových buniek (Mills a kol., 2019, Gupta kol., 2019). Bunková plasticita znamená schopnosť bunky prispôsobiť sa meniacim sa okolnostiam. Pojem bunkové plasticity vytvoril niekoľko termínov pre popis buniek, ktoré sa nachádzajú medzi kompletnými epiteliálními a mezenchymálnymi fenotypmi. pEMT (parciálna EMT), hybridné EMT a zmiešané EMT (Bakir a kol., 2020). Dôležitosť bunkovej plasticity zdôrazňujú niektorí autori, ktorí dokázali že tzv. hybridné EMT bunky nie sú zmiešanými bunkami epiteliálneho a mezenchymálneho stavu, ale ide o dynamiku zmien buniek v hybridnom stave, ktorá poskytuje týmto bunkám zvýšený metastatický potenciál (Kröger a kol., 2019).

1.3. Metastatický proces nádoru

Proces nádorového metastázovania je šírenie nádorových buniek do tkanív a orgánov mimo miesta, kde nádor vznikol. Tvorba nových sekundárnych nádorov je komplexný a unikátny dej. Tento proces diseminácie vzniká na základe zvyšujúcej sa heterogenity nádorovej populácie so vznikom subklónov, ktoré selektívnym tlakom získali vlastnosti potrebné pre realizáciu metastatického rozsevu (Zámečník a kol., 2019).

Proces sa skladá zo súboru po sebe nasledujúcich dejov, ktoré musia byť dokončené, aby nádorová bunka úspešne metastázovala. Ide o tzv. metastatickú kaskádu. Pri nej majú zásadný význam zmeny adhézie bunka-bunka a bunka-matrix. Dôležitým dejom pre vznik metastázy je vznik tzv. premetastatickej niky (PMN)-oblasti alebo určitého stromálneho mikroprostredia, ktoré neobsahuje nádorové bunky, ale svojimi vlastnosťami podporuje prežitie a kolonizáciu nádorových buniek do tejto špecifickej oblasti. Pre tieto deje je dôležitá komunikácia medzi bunkami nádoru a stromálnymi bunkami PMN. V tomto intercelulárnom predávaní informácií na molekulárnej bunkovej úrovni zohrávajú dôležitú úlohu exozómy a tzv. exozomálna komunikácia (viz kapitoly 1.3.2 a 1.3.3).

1.3.1. Metastatická kaskáda

Dej sa skladá zo 4 fáz:

1. migrácia nádorových buniek z primárneho ložiska
2. prestup cez cievnu stenu obehu (intravazácia) a prežitie bunky v cirkulácii

3. extravazácia-vystúpenie cez cievnu stenu do strómy cielového miesta
4. metastatická kolonizácia-zahájenie rastu metastatického ložiska

1.3.1.1. Lokálna migrácia nádorových buniek z primárneho tumoru

Pre uvoľnenie buniek z nádorovej masy sú dôležité zmeny expresie a funkcie molekúl zodpovedné za medzibunkové väzby (cadheríny) a väzbu buniek k extracelulárnej matrix (integríny). Mechanizmom podporujúcim tento dej je aktivácia génov, exprimujúcich integríny, ktoré podporujú proliferáciu, a migráciu nádorových buniek (integrín alfavBeta3), a potlačenie génov exprimujúcich integríny, ktoré sú zodpovedné za adhéziu nádorových buniek k extracelulárnej matrix (integrín alfa 2 beta1) (Zámečník a kol., 2019). Pre preniknutie buniek bariérami musia nádorové bunky aktívne degradovať extracelulárnu matrix a tak vytvárať cestu pre ich migráciu, pomocou proteolytických enzýmov, ako sú matrixové metaloproteinázy (MMP), alebo katepsíny. V tomto deji zohrávu úlohu tiež už spomínaná EMT. Neoplastické bunky zvyšujú tým svoju pohyblivosť. Motilitu buniek ovplyvňujú tiež zmeny v usporiadaní cytoskeletálních proteínov, a tiež receptorové interakcie s molekulami extracelulárnej matrix, ktorou migrujúca bunka preniká. Nádorové bunky s takými vlastnosťami opúšťajú primárny nádor, rozrušujú bazálnu membránu, prenikajú interstíciom a migrujú k bazálnej membráne ciev (Zámečník a kol., 2019, Nataraj a kol., 2021).

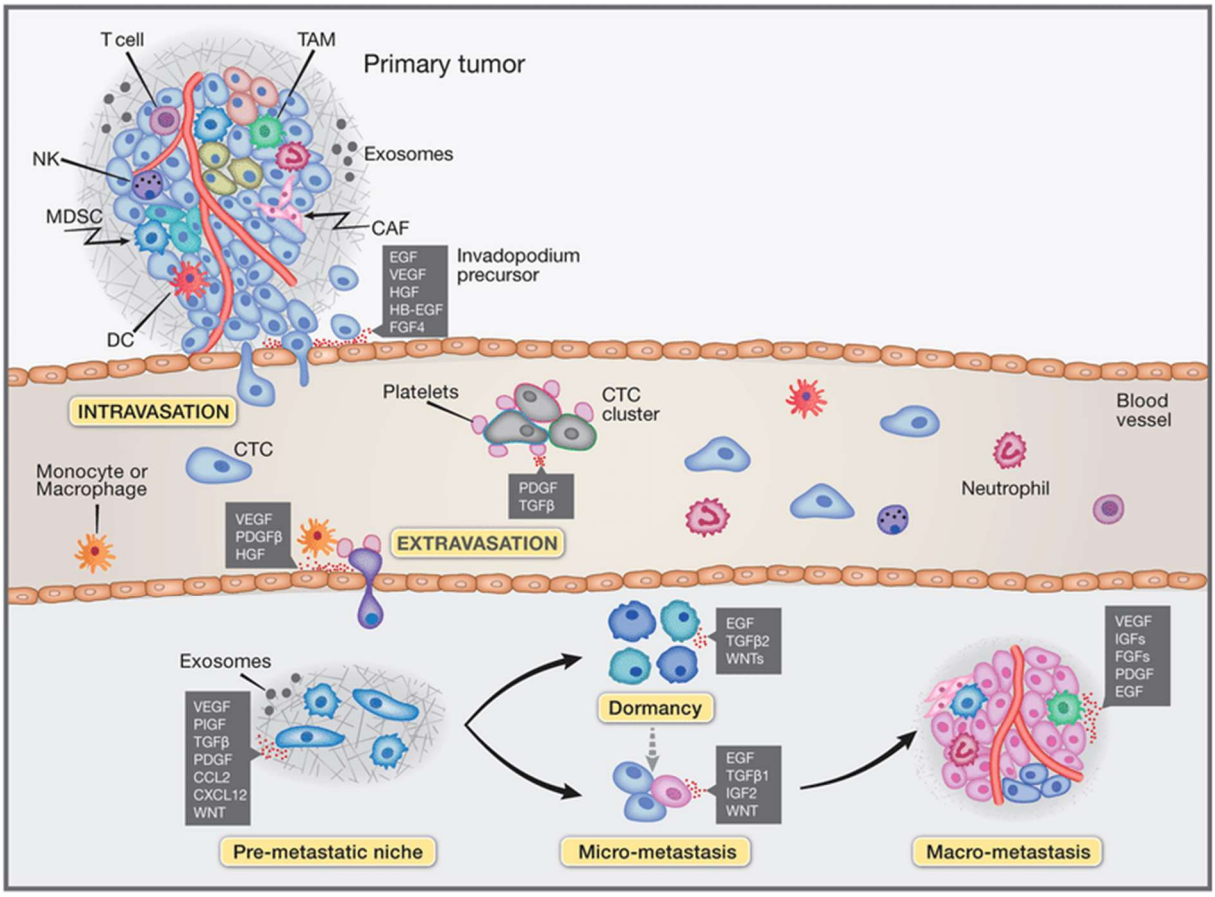
1.3.1.2. Intravazácia (vaskulárna diseminácia) a extravazácia nádorových buniek

Nádorové bunky prenikajú do krvných alebo lymfatických ciev tým, že najprv proteolyticky zničia bazálnu membránu cievy a potom prenikajú medzi endotéliami do cievneho riečišťa. Cievy nádoru nemajú dobre vytvorené endoteliální spoje a nemajú podporné pericyty, ani bazálnu membránu, čo spôsobuje ich zvýšenú priestupnosť. Invázia do týchto ciev a ich ďalšie šírenie na iné miesto je pre nádorové bunky ľahšie. Nádorové bunky tvoria v cievach mikrotromby, ktoré pomáhajú neoplastických bunkám lepšie prežitie, ale i jednoduchšiu extravazáciu mimo cievy a založenie nového ložiska (Zámečník a kol., 2019).

Interakcie endotelu a nádorových buniek v krvnom lúmene je rozdelená na počiatočnú fázu tzv. fázu “dockingu“, kedy bunky nádoru tvoria voľné väzby s endotelom, a pozdnú tzv. “locking“ fázu, kde ide o pevnú adhéziu. Najpevnejšie adhézie medzi bunkami sprostredkovávajú integrínové receptory. V tomto procese sa uplatňuje selektívna väzba medzi nádorovou bunkou a receptorom na endoteliálnej bunke (tzv.“ homing“) (Zámečník a kol., 2019)

1.3.1.3. Metastatická kolonizácia nádorových buniek

Ide o poslednú, najnáročnejšiu etapu kaskády, kedy bunky majú schopnosť prežívať a rásť vo zvláštnom mikroprostredí (premetastatická nika-PMN), ktoré jej špeciálne pomáha a podporuje ju. Ide o PMN opísanú nižšie (Zámečník a kol., 2019, Nataraj a kol., 2021) (obr.2).



Obr. 2 Schematický model metastatického šírenia nádorových buniek (zdroj: prevzaté z „Nataraj a kol**.,** 2021“)

Primárne nádorové bunky sú zabudované v mikroprostredí tvoreným stromálnymi bunkami ako sú fibroblasty asociované s karcinomom (CAF), myeloidné supresorové bunky (MDSC), dendritické bunky (DC), prirodzené NK bunky, T bunky, supresorové bunky, tumor asociované makrofágy (TAM), a neutrofily s krvnými cievami. Vylučovanie nádorových buniek a ich invázia cez tkanivové bariery umožňuje vytvorenie cirkulujúcich nádorových buniek (CTC) a dokončenie procesu intravazácie. V krvných cievach sú zhluky CTC a osamotené bunky chránené krvnými doštičkami, ktoré pomáhajú aj extravazácii. Solubilné faktory a exozómy vylučované nádorom spolupracujú na vytvorení premetastatickej niky. Mikrometastázy často prechádzajú dlhou fázou dormancie. Rastové faktory (zobrazené ako červené bodky) a cytokíny zahrnuté v každom kroku kaskády sú uvedené v odpovedajúcich rámčekoch. Použité skratky:

CCL2, C-C chemokinový ligand 2; CXCL123, chemokínový ligand 12 C-X-C; EGF, epidermalny rastový faktor; FGF, fibroblastický rastový faktor; HB-EGF, heparin viažucí „EGF like“ rastový faktor; HGF, hepatocytárny rastový faktor; IGF, inzulínu podobný rastový faktor; PDGF, platelet-derived growth factor; PIGF, placental growth factor; TGFβ, transforming growth factor beta; VEGF, vascular endothelial growth factor; WNTs, Wnt family members.

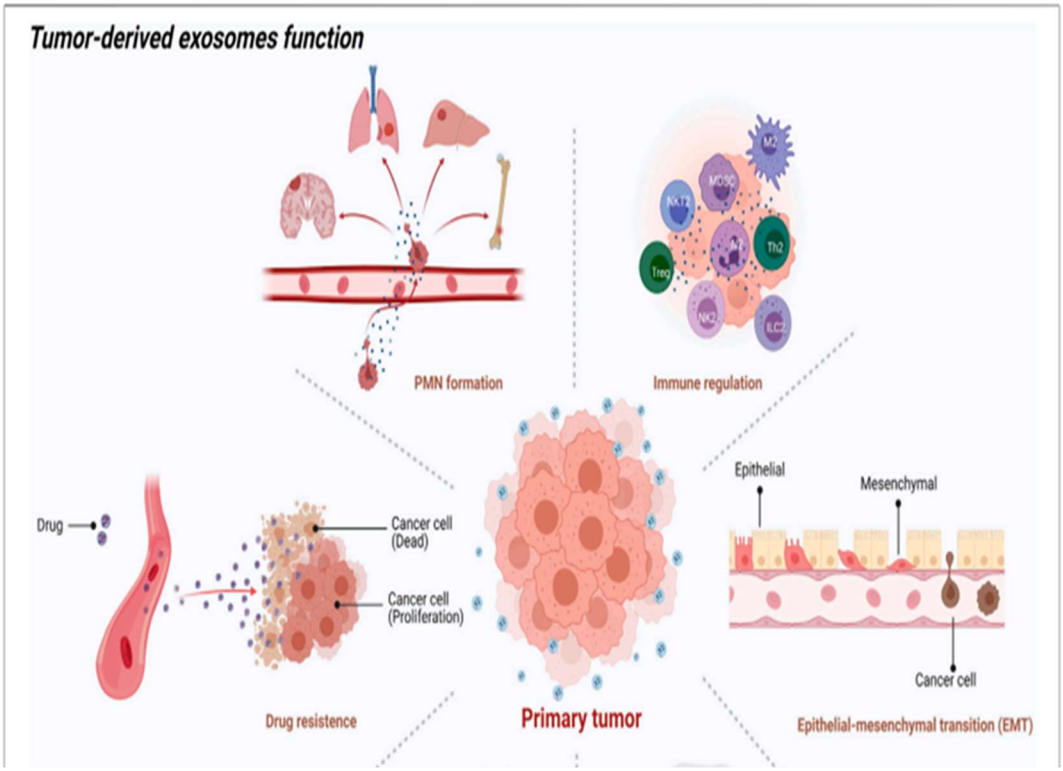
1.3.2 Exozómy a intercelulárna komunikácia

Úvodom k PMN je dôležité definovať pojem extracelulárne vezikuly (EVs), ktoré sú heterogénnou skupinou membránových štruktúr o rôznej veľkosti, ktoré sú vypudzované všetkými typmi buniek do extracelulárnych priestorov a majú veľa rôznych fyziologických a patofyziologických funkcií (Raposo a kol., 2013, Colombo a kol., 2014).

Rôzne typy EVs zahŕňajú exozómy, mikrovezikuly, apoptotické telieska, onkozómy a megazómy, ktoré sú charakterizované na základe ich biogenetickej ceste vzniku a na základe ich veľkosti. Exozómy majú endocytický pôvod a vznikli fúziou medzi multivezikulárnymi telieskami a plazmatickou membránou bunky. Sú podtypom extracelulárnych vezikul s priemerom 30-150 nm (Tai a kol., 2018, Rajagopal a kol., 2018, Kalluri a kol., 2003). Exosómy boli nájdené v krvi, moči, slinách a iných telových tekutinách (Kalluri a kol., 2009). Ako kľúčový hráč v intercelulárnej komunikácii, exozómy prenášajú informácie cez ich náklad - sú dôležité pre výmenu lipidov a proteínov a genetickej informácie medzi bunkami pomocou mRNA, DNA a miRNA (Dai a kol., 2020). Hrajú hlavnú úlohu v progresii zhubného nádoru (Mathivanan a kol., 2012, Hannafon a kol., 2013). Exozómy odvodené od tumoru („tumor-derived“ exozómy - TDE), sú aktívne produkované nádorovými bunkami, ktoré ich využívajú k podpore a rastu tumoru. Sú nosičmi informácií, prenášajúce molekulárne a genetické

správy z nádorových buniek do normálnych alebo iných abnormálnych buniek sídliacich v blízkych alebo vzdialených miestach.

TDE nesú náklad, ktorý sčasti napodobňuje obsah rodičovských buniek a sú rovnako potenciálne zaujímavé ako neinvazívne biomarkery rakoviny (Therasa a kol., 2016). Niektoré štúdie dokázali, že TDE sa zapájajú do výmeny genetickej informácie medzi nádorovými bunkami s výsledkom produkcie početných nových krvných ciev, ktoré podporujú rast nádoru a inváziu (Mears a kol., 2004, Vaksman a kol., 2014). Tiež ovplyvňujú EMT (Rahman a kol., 2016, Cai a kol., 2021, Gaballa a kol., 2020) ako javu, ktorý zvyšuje agresívnosť nádoru cestou podpory metastázovania nádorových buniek (Rahman a kol., 2016, Huang a kol., 2020, Cai a kol., 2021, Gaballa a kol., 2020). Tým všetkým podporujú progresiu nádoru (Paskeh a kol., 2022, Ramírez-Ricardo a kol., 2020, Yang a kol., 2021). Funkcia TDE je zhrnutá v obr. 3.

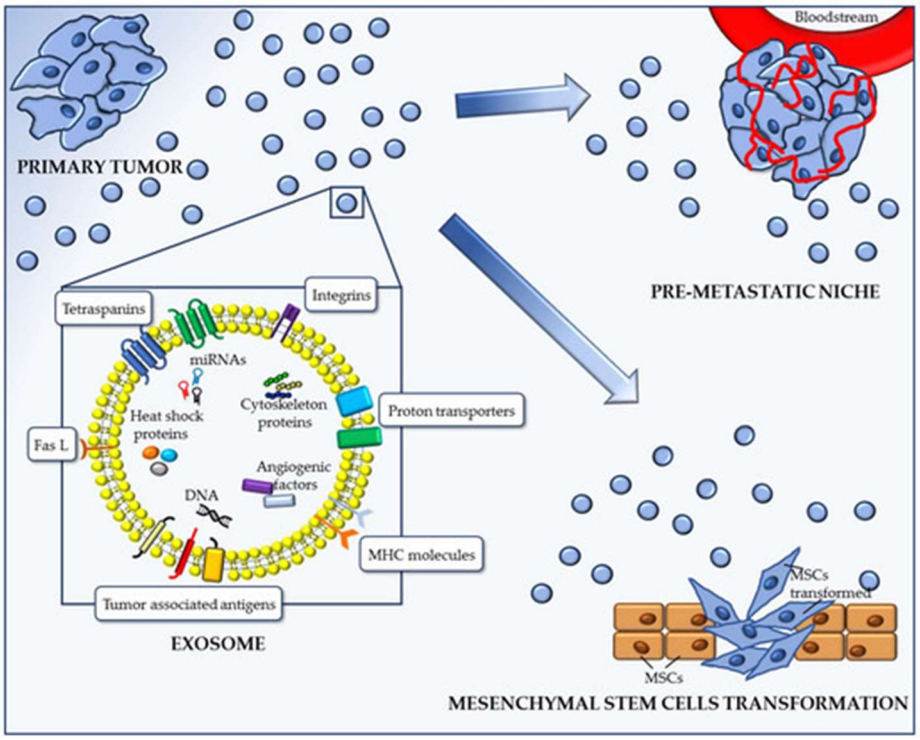


Obr.3 TDE a ich funkcie. TDE sa zapájajú okrem už spomínanej tvorby PMN aj do ďalších funkcií ako regulácia imunity, ovplyvnenie EMT, rezistencia na onkologické chemoterapeutiká pomocou ovplyvňovania proliferácie nádorových buniek.(zdroj: prevzaté z“ Yang a kol., 2021“)

1.3.3. Tzv. premetastatická „nika“ (PMN) ako prekurzor metastáz

Vzniku vzdialenej metastázy nádoru predchádza vznik tzv. predchodcu metastázy, ktorým je tzv. premetastatická nika. Ide o mikroprostredie, ktoré je vhodné pre nádorovú inváziu, vytrvanie a proliferáciu malígnych buniek, s cieľom vývinu do metastázy tumoru (Psaila a kol., 2009). Pojem premetastatická nika použil poprvé Kaplan se spolupracovníkmi (Kaplan a kol., 2006), a potom ďalší autori (Psaila a kol., 2009). Definovali PMN ako oblasť podporujúcu výživu nádorových buniek, avšak bez prítomnosť neoplastických buniek. Napokon prvý autor, ktorý dal základ koncepcii PMN a metastázovania primárneho nádoru bol Paget vo svojej teórii „semienka

a pôdy“ (Paget a kol., 2008). Dôležité prirovnanie a tvrdenie je:“ Keď rastlina rastie rozširuje svoje semienka. Jej semená sú nesené všetkými smermi, ale môžu žiť a rásť len vtedy, keď padnú na úrodnú pohostinnú pôdu“. Primárny nádor (rastlina) produkuje semienka (nádorové bunky), ktoré zakladajú vzdialené metastázy v oblastiach na to vhodných a predurčených (úrodná pôda) (Paget a kol., 1889, Fidler a kol., 2008). Niektoré práce ukazujú, že formácia pre-metastatické niky (PMN) závisí vo veľkej miere na už spomínaných TDE (Kaplan a kol., 2005, Peinado a kol., 2011, Sceneay a kol., 2013, Peinado a kol., 2012, Hood a kol., 2011, Spugnini a kol., 2018) (obr. 4).



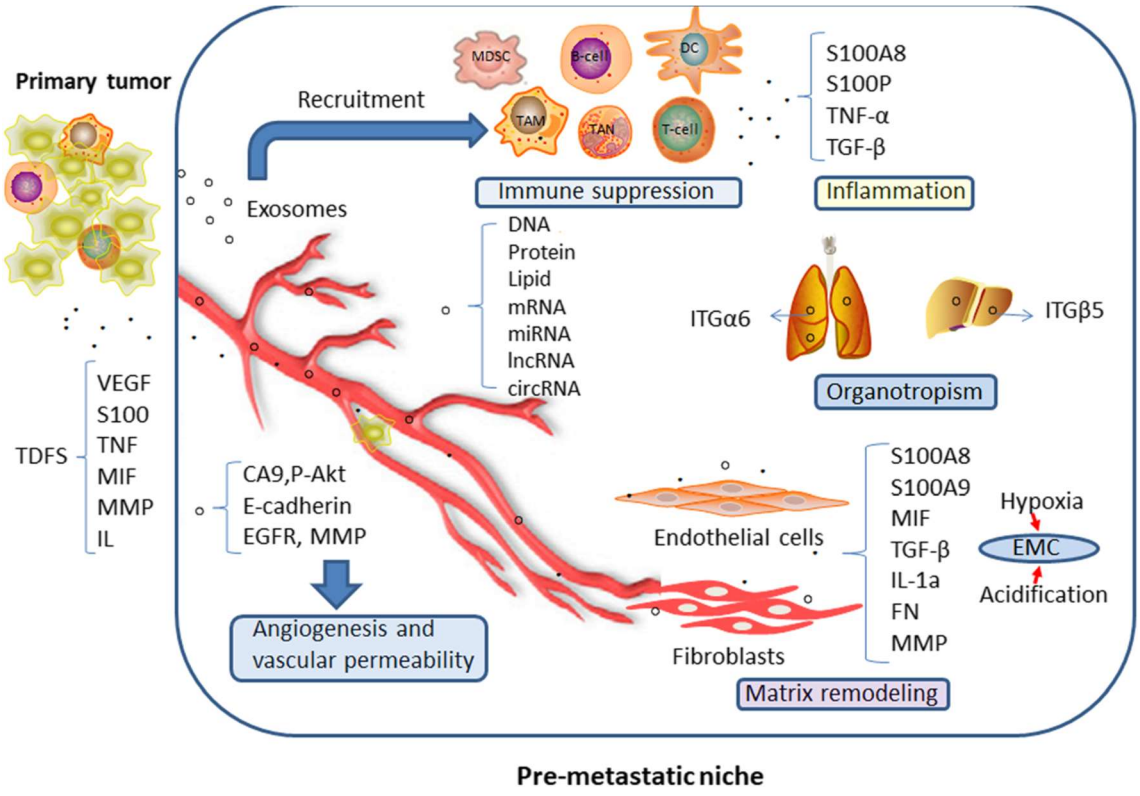
Obr.4 Schematický obrázok znázorňuje exozomálny transport z primárneho tumoru, ktorý ovplyvňuje vznik PMN, a má vplyv na EMT. (zdroj: prevzaté z „Spugnini a kol., 2018 “)

1.3.3.1. Význam TDE pri formovaní premetastatickej niky (PMN)

TDE produkované nádorovými a stromálnymi bunkami sa cirkuláciou dostávajú do vzdialených orgánov, kde spúšťajú kaskádu lokálnych zmien zahrňujúcich zvýšenie vaskulárnej permeability (Huang a kol., 2009, Araldi a kol., 2012), remodeláciu modifikovanej extracellularnej matrix (ECM), atrahovanie buniek kostnej drene (Kitamura a kol., 2015, Wang a kol., 2019), angiogenézu (Gupta a kol., 2007, Huang a kol., 2009, Zhou a kol., 2014), a imunosupresiu (Chen a kol., 2011, Tacke a kol., 2012, Giles a kol., 2016). Zvýšenie vaskulárnej permeability podporuje extravazáciu nádorových buniek a uľahčuje metastázovanie zničením adhezívnych molekúl medzi endoteliálnymi bunkami. Ďalšou kľúčovou udalosťou pri tvorbe PMN je remodelácia ECM, ktorá je charakterizovaná depozíciou nových komponent ECM a expresiou enzýmov súvisiacich s modifikáciou ECM. Remodelovaná ECM zvyšuje tuhosť matrice, čo ovplyvňuje vlastnosti nádorových buniek (Erler a kol., 2009, Cox a kol., 2013, Wu a kol., 2021).

Dalšou funkciou TDE je mobilizácia buniek kostnej drene, ktoré sa v cieľových orgánoch zúčastňujú angiogenézy a imunitných reakcií. (Dong a kol., 2021). TDE prispievajú k imunosupresívnemu mikroprostrediu PMN (Seubert a kol., 2015, Patel a kol., 2018). Mechanizmom vzniku tejto imunosupresívnej PMN je imunitný únik navodený pomocou tzv. „exosome-derived“PD-L1, ktoré sú prítomné vo vysokých hladinách v nádorovom tkanive, stromálnych bunkách a tumor asociovaných antigén-prezentujúcich bunkách (Boussiotis a kol., 2016, Dong a kol., 2002, Chen a kol., 2015, Gordon a kol., 2017). Dalším mechanizmom imunosupresie je vyplavovanie inhibítorov imunitných buniek. Nádorové exozómy spôsobujú atrahovanie „tumor-associated“ makrofágov (TAMs), tumor-asociovaných

neutrofilov (TAN), regulačných T (Treg) buniek a myeloid-derivovaných supresorových buniek (MDSC) na vzdialené sekundárne miesta (Liu a kol., 2016). Tieto imunitné bunky inhibujú protinádorovú aktivitu ostatných buniek (McAllister a kol., 2014, Liu a kol., 2016). Všetky tieto opísané deje zhrňuje obr.5.



Obr.5 Grafické prehľadné zhrnutie funkcií TDE pri tvorbe PMN. (zdroj: prevzaté z „Guo a kol., 2019 “)

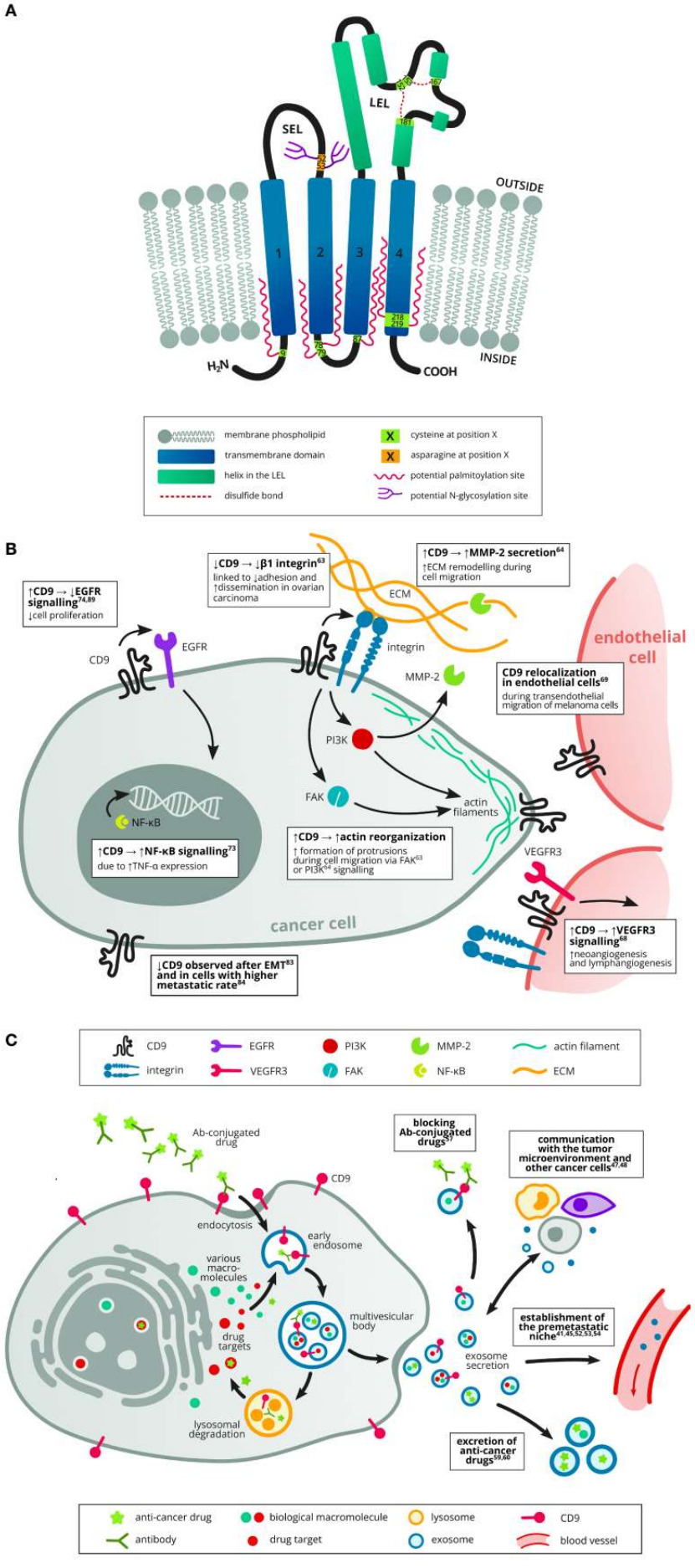
1.4. Mnohostranná úloha tetraspanínu CD9 u nádorov

Súčasný výskum TNBCs je zameraný na identifikáciu nových proteínov ako kandidátov efektívnej cieľovej onkologickej terapie (Allison a kol., 2019) alebo ako nových prognostických markerov.

Tetraspanín CD9, tiež známy ako TSPAN29, alebo proteín 1 súvisiaci s motolitou, je členom z rodiny transmembránových proteínov, ktorá sa vyznačuje štyrmi transmembránovými doménami, dvoma extracelularnými slučkami a krátkymi intracelularnými N- a C- koncami (Obr. 6A) (Reyes a kol., 2018, Brosseau a kol., 2018). Rovnako ako ostatné tetraspaníny môže podstúpiť CD9 palmitoylaciu na každom z jeho membránových proximálnych cysteínov, ktorá ovplyvňuje jeho interakcie s ďalšími partnermi (Yang a kol., 2006). Tetraspaníny všeobecne tvoria mikrodomény obohatené tetraspanínom (TEM) v bunkových membránach. V rámci týchto domén interagujú s rôznymi transmembránovými a intercelularnými partnermi, vrátane ďalších tetraspanínov, integrínov, proteáz, imunoglobulínov a intracelulárnych signálnych proteínov (Rubinstein a kol., 2013). Preto biologické účinky CD9 závisia na týchto dynamických interakciách vo vnútri TEM (Reyes a kol., 2018, Rubinstein a kol., 2013). Bol navrhnutý tzv. model reťazenia, pri ktorom sa vytvárajú komplexy CD9/EWI-F, ktoré môžu vysvetliť výskyt týchto TEM (Hemler a kol., 2005). Okrem tetraspanínov (napr. CD63, CD81, CD151 a TSPAN4), CD9 interaguje s početnými transmembránovými proteínmi, ako sú integríny (napr. CD49c/ITGA3 a CD29) (Reyes a kol., 2018, Soung a kol., 2017), proteíny imunoglobulínovej rodiny (napr. EWI-F/PTGFRN a EWI-2/IGSF8) (Reyes a kol., 2018, Radford a kol., 1996), heparin viazací rastový faktor faktor podobný EGF (Wang a kol., 2011), a metaloproteáza ADAM17 (A Disintegrin

And Metalloproteinase 17) (Iwamoto a kol., 1994). CD9 je tiež schopný interakcie s ďalšími proteínmi ako CD19, CD46 a CD117 (Machado-Pineda a kol., 2018, Anzai a kol., 2002, Horvath a kol., 1998). Veľmi dôležitú úlohu hrajú interakcie s intracelulárními signálnymi molekulami (Yang a kol., 2006). Ide hlavne o interakcie s malými GTPázami z rodiny Rho, ktoré ovplyvňujú aktínový cytoskelet (Gutierrez-Lopez a kol., 2011, Herr a kol., 2014), ERM proteíny, ktoré sprostredkúvajú väzbu s cytoskeletom a PKC (Arnaud a kol., 2015), ktorý reguluje funkciu širokej škály proteínov. Je jasné, že tetraspanín CD9 hrá komplexnú úlohu ako vo fyziologických podmienkach tak i u veľa chorôb vrátane solídnych nádorov (obrázok 6B).

Obr. 6 CD9. Štruktúra CD9 a jeho úloha pri nádoroch, vrátane exozomálneho transportu (A) Proteín CD9 sa skladá zo štyroch transmembránových domén, krátka (EC1,SEL) a dlhá (EC2,LEL) extracelulárna slučka, krátka intracelulárna slučka a krátke intracelulárne N- a C- konce. V LEL sú dva disulfidové mosty, z nich každý obsahuje jeden cystein, typický znak rodiny tetraspaninov. (B) CD9 sa podieľa na mechanizmoch podporujúcich potlačovanie rastu nádoru. Boli popísané štúdie o úlohe migrácie nádorových buniek a invázie, napr. ovplyvnením aktín-polymerizácie a reorganizácie na bunkových výbežkoch, alebo zvýšením produkcie proteinázy MMP-2, ktorý rozkladá zložky ECM behom bunkové invázie. Zvýšená expresia CD9 bola spojená so zvýšenou signalizáciou v protumorigennnej NF-k B dráhe. Zvýšená expresia CD9 zoslabuje signalizáciu EGFR a tým potlačuje bunkovú proliferáciu. Inde popísali vyššiu prítomnosť metastáz v bunkách so zníženou expresiou CD9. Down regulácia CD9 bola pozorovaná u buniek, ktoré podstúpili EMT. CD9 tiež ovplyvňuje neoangiogenézu nádoru podporou signalizácie VEGFR3 v endotéliách. Transendoteliálna migrácia nádorových buniek je podporovaná reorganizáciou štruktúry CD9 v miestach kontaktu (medzi endotelom a nádorovou bunkou). Exozómy transportujú náklad medzi bunkami v mikroprostredia nádoru, a tak umožňujú vzájomnú komunikáciu. Vytvárajú premetastatickú niku v cieľovom orgáne pred kolonizáciou. Exozómy podporujú liekovú rezistenciu niekoľkými mechanizmami (transportom liekov z nádorových buniek, alebo neutralizáciou konjugovanej protilátky drogy. (zdroj: prevzaté z „Ondruššek a kol., 2023“)


A
LEL
SEL
OUTSIDE
INSIDE
1
2
3
4
H_2N
COOH
membrane phospholipid
transmembrane domain
helix in the LEL
disulfide bond
cysteine at position X
asparagine at position X
potential palmitoylation site
potential N-glycosylation site
B
↓CD9 → ↓β1 integrin[63]
linked to ↓adhesion and ↑dissemination in ovarian carcinoma
↑CD9 → ↑MMP-2 secretion[64]
↑ECM remodelling during cell migration
↑CD9 → ↓EGFR signalling[74,89]
↓cell proliferation
ECM
CD9
EGFR
integrin
endothelial cell
MMP-2
CD9 relocalization in endothelial cells[69]
during transendothelial migration of melanoma cells
PI3K
FAK
actin filaments
NF-κB
↑CD9 → ↑NF-κB signalling[73]
due to ↑TNF-α expression
↑CD9 → ↑actin reorganization
↑ formation of protrusions during cell migration via FAK[63] or PI3K[64] signalling
VEGFR3
cancer cell
↑CD9 → ↑VEGFR3 signalling[68]
↑neoangiogenesis and lymphangiogenesis
↓CD9 observed after EMT[83] and in cells with higher metastatic rate[84]
C
CD9
EGFR
PI3K
MMP-2
actin filament
integrin
VEGFR3
FAK
NF-κB
ECM
Ab-conjugated drug
blocking Ab-conjugated drugs[57]
communication with the tumor microenvironment and other cancer cells[47,48]
CD9
endocytosis
early endosome
various macro-molecules
multivesicular body
drug targets
establishment of the premetastatic niche[47,45,52,53,54]
exosome secretion
lysosomal degradation
excretion of anti-cancer drugs[59,60]
anti-cancer drug
biological macromolecule
lysosome
CD9
antibody
drug target
exosome
blood vessel

CD9 moduluje bunkovú adhéziu, migráciu a proliferáciu buniek. Ovplyvňuje proces tvorby a stabilizácie bunkových membrán a proces bunkového vezikulárneho transportu vrátane exozómov (Rappa a kol., 2016). Významne sa uplatňuje v interakciách medzi nádorovými bunkami a stromálným mikroprostredím, ktoré podstatným spôsobom ovplyvňujú rast a metastázovanie nádorov (Ekström a kol., 2022, Yoshioka a kol., 2013).

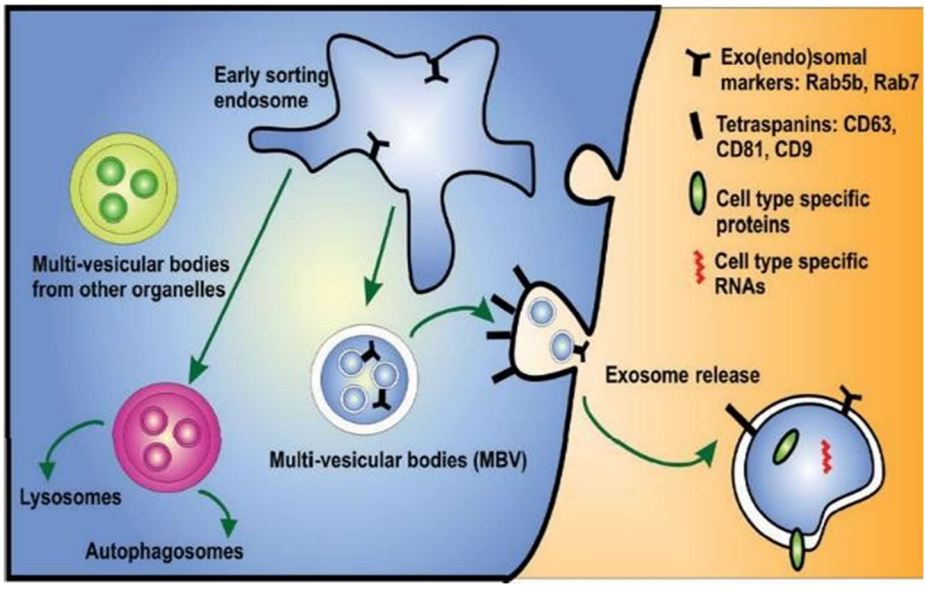
1.4.1 Fyziologické úlohy CD9

CD9 je kľúčovým regulátorom bunkovej adhézie v imunitnom systéme a hrá dôležitú úlohu vo fyziológii leukocytov a endoteliálních buniek, tiež i v krvotvorbe a pri zrážaní krvi. Ďalšími fyziologickými funkciami je fúzia spermií s vajíčkom (Miyado a kol., 2000), rast neuritov (Kagawa a kol., 1997) alebo myotubulov (Charrin a kol., 2014). Jednou z dôležitých funkcii CD9 je regulácia diferenciácie hematopoetických kmeňových buniek v kostnej dreni a kritické udalosti krvotvorby. Je potrebný pre megakaryocytárnu (Clay a kol., 2001), lymfoidnú a myeloidní diferenciáciu (Oritani a kol., 1996). Úloha CD9 v rôznych imunitných bunkách a jej význam pre zápal preskúmal Brosseau a spol (Brosseau a kol., 2018). CD9 hrá dôležitú úlohu v imunitnej synapse (Reyes a kol., 2018). CD9 ovplyvňuje vírusové (Sims a kol., 2018) a bakteriálne infekcie (Ventress a kol., 2016, Green a kol., 2011). V rámci TEM moduluje CD9 rôzne procesy vyvolané vírusom, zahrnúc membránové fúzie, pučanie a uvoľnenie vírusov. Sims a kol. dokázal, že exozómy môžu zosilniť vstup HIV 1 do ľudských T a monocytárných bunkových línií pomocou tetraspaninových proteínov CD81 a CD9 (Sims a kol., 2018).

1.4.2. CD9 v exozómoch a medzibunková komunikácia

Tetraspaníny sú bohatou súčasťou exozomálnej membrány, pričom CD9 je jedným z najčastejšie prítomných, spolu s CD63, CD81 a TSPAN8 (Lorico a kol., 2021, Mathivanan a kol., 2010). Dôležité je, že tetraspaníny môžu ovplyvniť zloženie exozómov prostredníctvom interakcií s ich väzbovými partnermi (obr.7).

Mechanizmy exozómovej biogenézy sú vysoko regulované pomocou niekoľkých odlišných ciest, vrátane ESCRT (endosomal sorting complexes required for transport) - závislé a ESCRT - nezávislé ceste (Tai a kol., 2018). Aj keď uvoľňovanie exozómov je fyziologický proces, jeho zvýšená rýchlosť a špecifický náklad pôsobia priaznivo pre onkogénnu progresiu a vznik metastáz (Rajagopal a kol., 2018). Exozomálna komunikácia nie je obmedzená len na nádorové bunky, ale tiež bola zobrazená v rôznych typoch buniek v mikroprostredí nádoru lokálne i vzdialene. Bioaktívne molekuly, vrátane CD9, odvodené od exozómov nádorových buniek a stromálnych buniek, poskytujú zásadné signály na výchovu rôznych buniek a remodeláciu architektúry nádoru (Miki a kol., 2018, Nigri a kol., 2022).



Obr.7 CD9 ovplyvňuje proces bunkového vezikularného transportu.

Zdroj: prevzate z“ Rappa a kol.,2015“)

1.4.3. CD9 v nádorových bunkách: Dr. Jekyll a pán Hyde

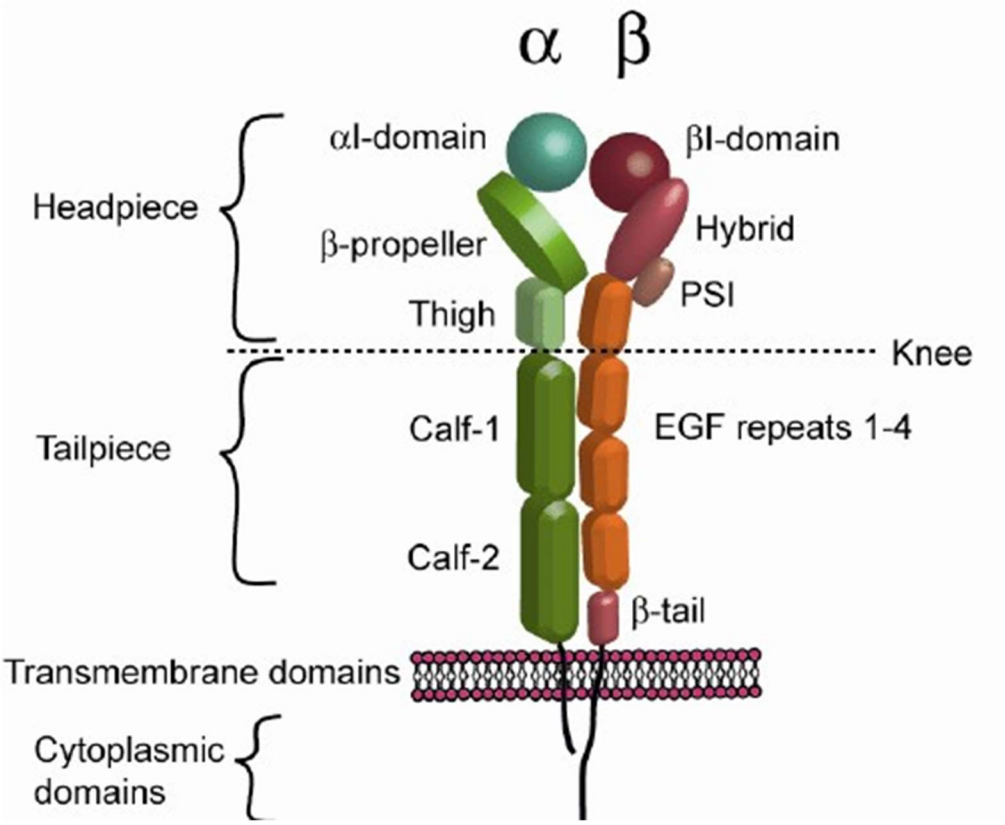
Expresia CD9 je deregulovaná v celej škále patologických procesov vrátane zhubných nádorov, ale presný mechanizmus , ktorý spôsobuje zmenu a súvisiace dôsledky nie sú dobre pochopené (Rubinstein a kol., 2013). Analýza väzbových miest v oblasti promótora génu CD9 identifikovala E2F, NFkB, SP1 a STAT3 ako hlavných transkripčných faktorov v procese karcinogenézy a prognózy ochorenia (Fishilevich a kol., 2017). CD9 hrá duálnu úlohu v nádorovej progresii, vykazujúc tumor -podporujúce i tumor-suprimujúce schopnosti (Ondruššek a kol. 2023). Naši spolupracovníci pomocou „flow“ cytometrickej analýzy v predošlej štúdii zistili, že znížená expresia CD9 and CD29 v bunkách karcinómu prsnej žlazy je spojená s EMT (Remšík a kol., 2018). V tejto práci sme si dali za cieľ vyšetriť CD9 a CD29 spoločne s E-cadherinem a vimentinem (EMT znaky) v súbore pacientiek s TNBC.

1.5. CD29 ako súčasť integrínov

Integriny sú veľkou rodinou transmembránových proteínov zapojených do bunkovej adhézie. Tvoria spojenie medzi intracelulárnymi cyskeletálnymi proteínmi a proteínmi extracelulárnej matrix. Integriny existujú ako heterodimery skladajúce sa z alfa a beta podjednotiek. Zapájajú sa do rôznych procesov ako je prenos signálu, migrácia buniek, proliferácia, diferenciácia a apoptóza (Hynes a kol., 2002, Giancotti a kol., 1999).

1.5.1 Štruktúra integrínov

Integríny sa skladajú z troch domén: extracelulárna doména, transmembránová doména a cytoplazmatický koniec. Veľká globulární extracelulárna doména je zodpovedná za väzbu ligandu, kým transmembránová oblasť je jednopriechodová a-helix. Cytoplazmatické konce sú krátke neštruktúrované oblasti, ktoré tvoria disulfidové mostíky medzi alfa a beta proteínmi a tiež interagujú s adaptorovými proteínmi, ktoré sú spojené s cytoskeletom. Integrinové komplexy sú schopné signalizovať obojsmerne. Väzba ligandu na vonkajšej strane bunky môže vyvolať intracelulárnu odpoveď, ako je reorganizácia cytoskeletu (Giancotti a kol., 1999) (Obr. 8).



Obr. 8 Schematické znázornenie integrínovej štruktúry všeobecne. Integrínové alfa a beta podjednotky sú zložené z extracelulárních, transmembránových a intracelulárních domén. Veľká extracelulárna doména može byť rozdelená na niekoľko menších domén. Nie všetky integrínové heterodiméry obsahujú alfa I doménu. (zdroj: prevzaté z „Nevo a kol., 2010“)

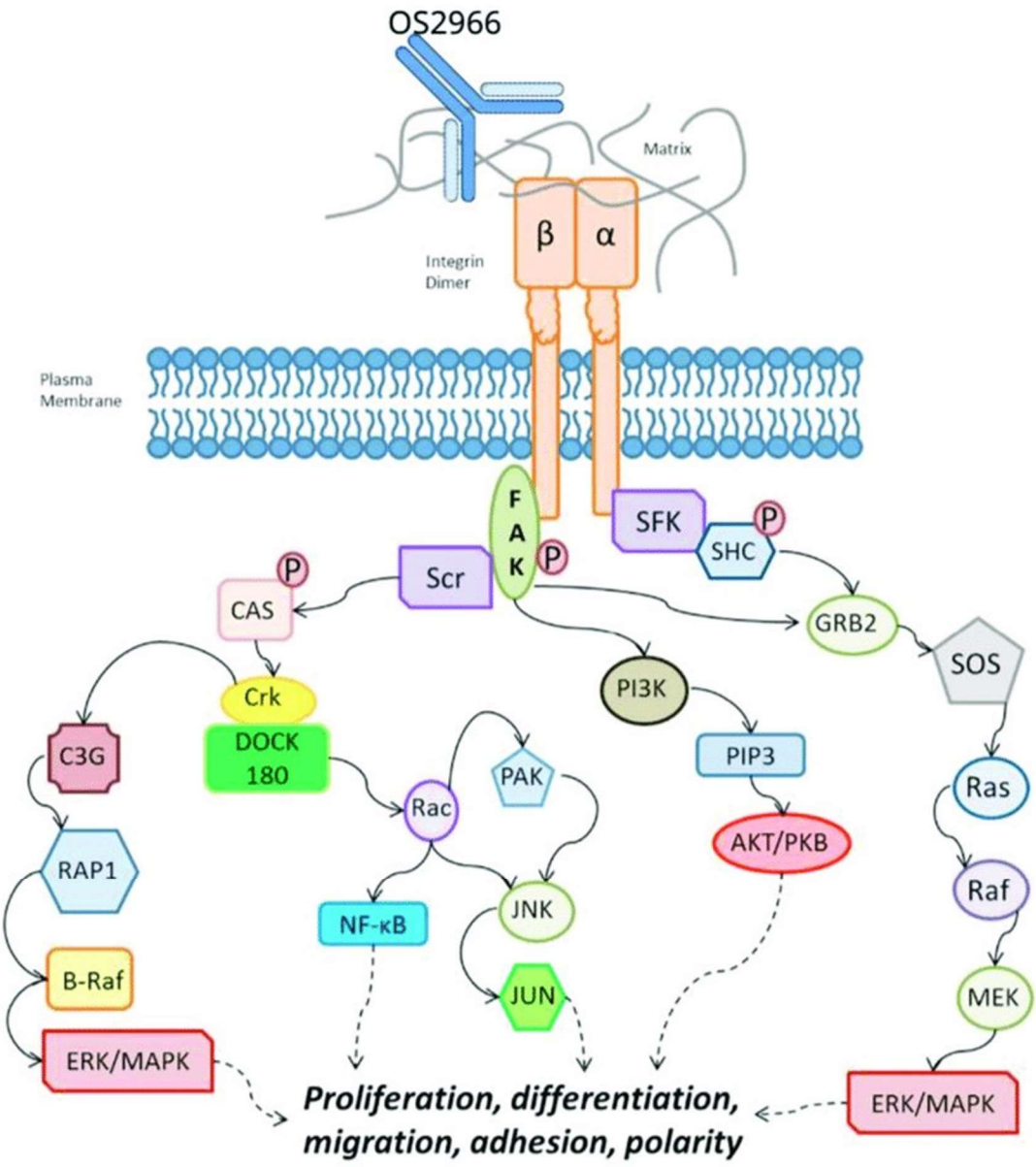
1.5.2. Podjednotka integrinu beta 1 (ITGB1, alias CD29)

CD29 známy ako integrin beta-1 je povrchový bunkový proteínový receptor kódovaný genóm ITGB1 patriaci do skupiny kolagénových receptorov, je aj tzv.markerom EMT(Geng a kol., 2013). CD29 reguluje rôzne biologické procesy a hrá rôzne signalizačné úlohy pri normálnom vývoji tkanív i pri ochoreniach. Medzi biologické procesy, ktoré ovplyvňuje, patrí bunková proliferácia, prežívanie buniek a ich migráciu (Juliano a kol., 1993, Clark a kol., 1995). CD29 je ovplyvňované hlavne proteínom glukózového transportu v nádorových bunkách tzv. Glut 1 (Oh Sunhwa a kol., 2017).

1.5.3. Interakcie medzi integrínmi a transmembránovými receptormi

V týchto procesoch sa okrem beta1 integrínu uplatňuje beta3 integrín a EGFR. Integríny indukujú ligand - nezávislú transaktivaciu EGFR (Moro a kol., 1998) a súčasne udržujú signalizáciu EGFR z plazmatické membrány do cytoskeletu (Azimifar a kol., 2012, Balanis a kol., 2011) a do jadra buniek (Cabodi a kol., 2009). Integrin/EGFR je kľúčový prvok pre šírenie signálnych dráh, ktoré kontrolujú biologické správanie nádorových buniek (Cabodi a kol., 2010, Desgrosellier a kol., 1997). Beta1 integrin je potrebný pre signalizáciu EGFR v neoplastických bunkách, a veľmi dôležitý pre rast buniek nádorov pľúc a prsnej žľazy, inváziu a rezistenciu buniek ku špecifickým liekom EGFR (Morello a kol., 2011). CD29 udržuje aktivity RTK a stimuluje signalizáciu IL-3, a následne ovplyvňuje bunkový cyklus endotélií (Defilippi a kol., 2005), a tvorbu krvných ciev v nádoroch (Uberti a kol., 2010).

Ďalší príklad spolupráce medzi integrínmi a transmembránovými receptormi je tzv. TGF-beta rodina. Remodelácia kolagénu, fibróza a hojenie rán, a tiež EMT a migrácia a invázia nádorových buniek sú javy na ktorých spolupracujú integrín a TGF beta. TGF-beta pomocou ADAM17 sprostredkuje uvoľňovanie rastového faktora a zosilňuje onkogénnu signalizáciu HER2 indukciou zhlukovania heterodimerov HER2/EGFR s integrinom beta1 (Wang a kol., 2009). Integriny môžu riadiť aj endozomálny prenos ďalších receptorov (Caswell a kol., 2009), ako EGFR (Morello a kol., 2011) a VEGFR2 (Ivaska a kol., 2011). Schematický obrázok (Obr. 9) ilustruje CD29 a dráhy a molekuly, ktoré ovplyvňuje (Chibueze a kol., 2021).



Obr. 9 Obrázok ilustruje najdôležitejšie cesty pôsobenia β-1 integrine/CD29 receptoru. Medzi kľúčové cesty, ktoré sú riadené pomocou CD29 patria charakteristiky nádora a biologické schopnosti získané viacstupňovým vývojom nádora ku ktorím patrí: signalizácia rastových faktorov, ktoré ovplyvňujú prežitie a proliferáciu nádora, invázia a metastazovanie, angiogenéza a vaskularizácia, imunitná reakcia, a rezistencia na konvenčnú terapiu (zvýšenie prežitia, EMT, zosilnená reakcia na stres). (zdroj: prevzaté z:“ Chibueze a kol., 2021“)

2. Ciele práce

1. Sledovať kvalitatívne a kvantitatívne imunohistochemickú expresiu proteínov CD9 a CD29 v centre a na periférii nádoru, v strome nádoru ako mikroprostredia, ktoré ovplyvňuje nádorové bunky.
2. Analyzovať expresiu epitelových a mezenchymálnych markerov E-cadherínu, a vimentínu pre definíciu javu EMT nádoru, ktorý hrá dôležitú úlohu pri invázii a tvorbe metastáz nádoru. EMT je aj jednou z príčin vzniku rezistencie na liečbu.
3. Štatisticky vyhodnotit tieto údaje s ďalšími charakteristikami nádoru v našom súbore ako je histologický grade, proliferačná aktivita v podobe proliferačného markera Ki-67, rozsah nádorovej choroby podľa patologického TNM systému a prežívanie pacietiek.
4. V nezávislom súbore z Masarykova onkologického ústavu vyhodnotiť expresiu proteínov CD97, p65, HLA-DR a ITGA3.

3. Materiál a metodika

Vyšetrili sme súbor 75 **„triple“ negatívnych karcinómov** z bioptického materiálu z kvadrantektómie alebo mastektómie pacientiek vyšetrených na našom pracovisku patológie CGB laboratória v časovom období od roku 2013 do 2022. Pacientky boli vo vekovom rozmedzí 38 do 81 let, s patologickým **štádiom** tumoru od **pT1b do pT4**, s **histologickým grade II-III**, dle Bloom-Richardson systému. Deväť pacientiek podstúpilo neoadjuvantnú chemoterapiu (väčšina pacientiek schéma: antracyklíny, cyklofosfamid a taxány, 1 pacientka: carboplatina + gemcitabine, 2 pacientky: capecitabine) a boli vyradené zo štatistickéj analýzy. U 18 pacientiek súboru bolo zistené metastatické postihnutie regionálnych lymfatických uzlín. V súbore bolo zastúpených 62 invazívnych karcinómov, NST typ, a ďalšie zvláštnejšie podtypy karcinómov s takýmto zastúpením: 8 apokrinných karcinómov, 2 adenoidne-cystické karcinómy, 1 salivary like“ karcinóm, bez bližšej špecifikácie (NST) a 2 adenoskvamózne karcinómy. Triple negatívne karcinómy boli definované ako karcinómy so súčasnou imunohistochemickou negativitou estrogenového a progesteronového receptoru a Her 2/neu, vrátane negativity genetického vyšetrenia FISH. Získané vzorky boli fixované v 10% formalíne a zaliate do parafínu. Tkanivové bloky zaliate v parafíne boli narezané na 2-3 mikrometrové rezy a zafarbené pomocou hematoxylín-eosínu. Proteín bol detegovaný na tkanivových rezoch študovaných nádorov metódou nepriamej imunohistochémie s použitím nasledujúcich monoklonálních protilátok: králičia anti CD9 (klon EPR 2949, v riedení 1:2000; Abcam), králičia anti CD29 (klon EP1041Y, v riedení 1:2000; Abcam), a nasledujúcich protilátok ako markerov pomáhajúcich definovať EMT: myší anti E cadherín (klon NCH-38,

v riedení 1:50, Dako), myší anti Vimentín (klon V9, v riedení 1:100, Dako). Imunohistochemické hodnotenie bolo prevedené kvalitatívne-kvantitatívne pomocou H (histo) skóre, stanovenom násobením percenta pozitívnych buniek a intenzity farbenia : 0 - žiadna expresia, 1+ mierna intenzita, 2+ stredná intenzita, 3+ silná intenzita.

Expresia bola sledovaná v centre nádoru a jej periférii, a to ako v nádorových bunkách, tak v stróme, a ďalej v okolitom nenádorovom tkanive prsnej žľazy. U pacientiek s prítomnosťou lymfatických metastáz bola porovnávaná imunohistochemická expresia CD9 a CD29 v primárnom tumore i v metastatických uzlinových ložiskách. Výsledky boli vyhodnotené pomocou Mann-Whitney U testu, Wilcoxonovho testu, Spearmanovho korelačného koeficientu a Kaplan Meier analýzou prežívania s log rank testom (STATISTICA 12,TIBCO Software).

Zvlášť udávame zoznam protilátok použitých v štúdii Kvokačková a kol., na ktorej sme sa podieľali interpretáciou imunohistochemickej expresie na vzorkách TMA (Tab č.1; viz 4.5 a Kvokačková a kol. 2023).

Tab č.1

protein	typ	klon	antigén retrieval	riedenie	Kat.č.	firma
CD97	kraličí	EPR4427	Flex	1:200	ab108368	Abcam
HLA-DR	myší	TAL. 1B5	Flex	1:100	M0746	Dako
ITGA3	králičí	poly	Flex	1:100	HPA008572	Merck
p65	myší	F-6	Flex	1:100	sc-8008	Santa Cruz

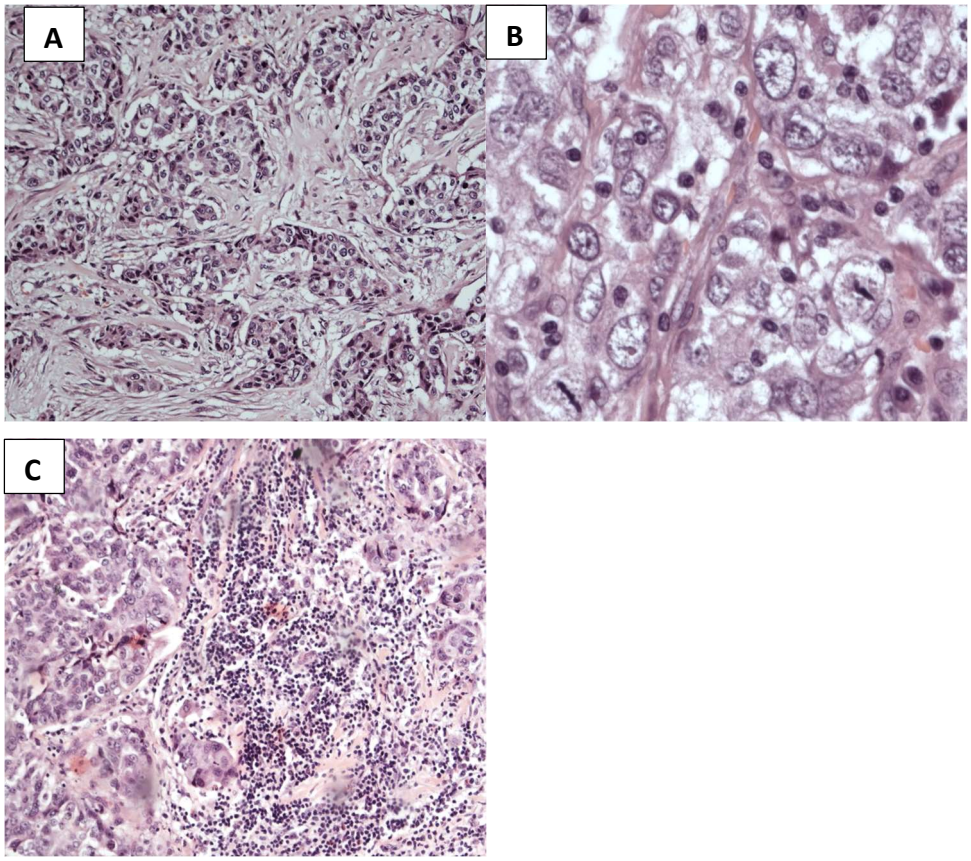
4. Výsledky

4.1. Charakteristika súboru pacientiek s TNBC

Náš súbor bol tvorený 75 „triple“ negatívnymi karcinómami (TNBC) z bioptického materiálu z kvadrantektómie alebo mastektómie pacientiek vyšetrených na našom pracovisku patológie EUC Laboratória CGB a.s. v časovom období od roku 2013 do 2022, vo vekovom rozmedzí od 38 do 81 rokov. Deväť pacientiek podstúpilo neoadjuvantnú chemoterapiu (väčšina pacientiek schéma: antracyklíny, cyklofosfamid a taxány, 1 pacientka: carboplatina + gemcitabine, 2 pacientky: capecitabine). Štatistický vyhodnotený súbor, v ktorom sme sledovali expresiu CD9, CD29, vimentínu, E-cadherínu a Ki67, bol zastúpený 66 pacientkami bez neoadjuvantnej chemoterapie. Pacientky s chemoterapiou sme vylúčili pre možnú modifikáciu expresie sledovaných proteínov. Klinicko-patologické informácie o našej kohorte pacientov sú zhrnuté v tabuľke č.2. Zo 66 pacientiek súboru sa u 18 vyskytol relaps choroby vo forme lokálnej rekurencie alebo v podobe vzdialených metastáz. Štyri pacientky mali metastatické postihnutie regionálnych lymfatických uzlín a 10 mali vzdialené metastázy do pečene, pľúc, metastatický rozsev v kožných jazvách, alebo generalizáciu tumoru do kostného skeletu a viscerálních orgánov. U 3 pacientiek bola prítomná nádorová duplicita v podobe kolorektálneho a ovariálného karcinómu, a Hodgkinovho lymfómu počas liečby. Desať pacientiek zomrelo na nádorové ochorenie (breast cancer specific survival, BCSS), a u 2 pacientiek bola smrť spôsobená inou nešpecifickou príčinou bez priamej súvislosti s ochorením (tabuľka č.2).

Všetky vyšetrované TNBC mali podobnú morfológiu s histologickým grade II-III, bez tvorby žliazok, väčšina s „high grade“ jadrami nádorových buniek

a zvýšenú proliferačnú aktivitu, niektoré s intenzívnou lymfocytárnou reakciou v okolitej stróme nádoru (obr. 10A-C).



Obr. 10 Morfológia TNBC. A - solídne čapy nádorových buniek, bez tvorby žliazok, B - detail nádorových buniek s „high grade“ vezikularnými jadrami, C - v stróme v tesnom susedstve neoplastických buniek intenzívne lymfocytárne infiltráty.

Tab č. 2 Klinicko-patologické dáta súboru TNBC (N=66)

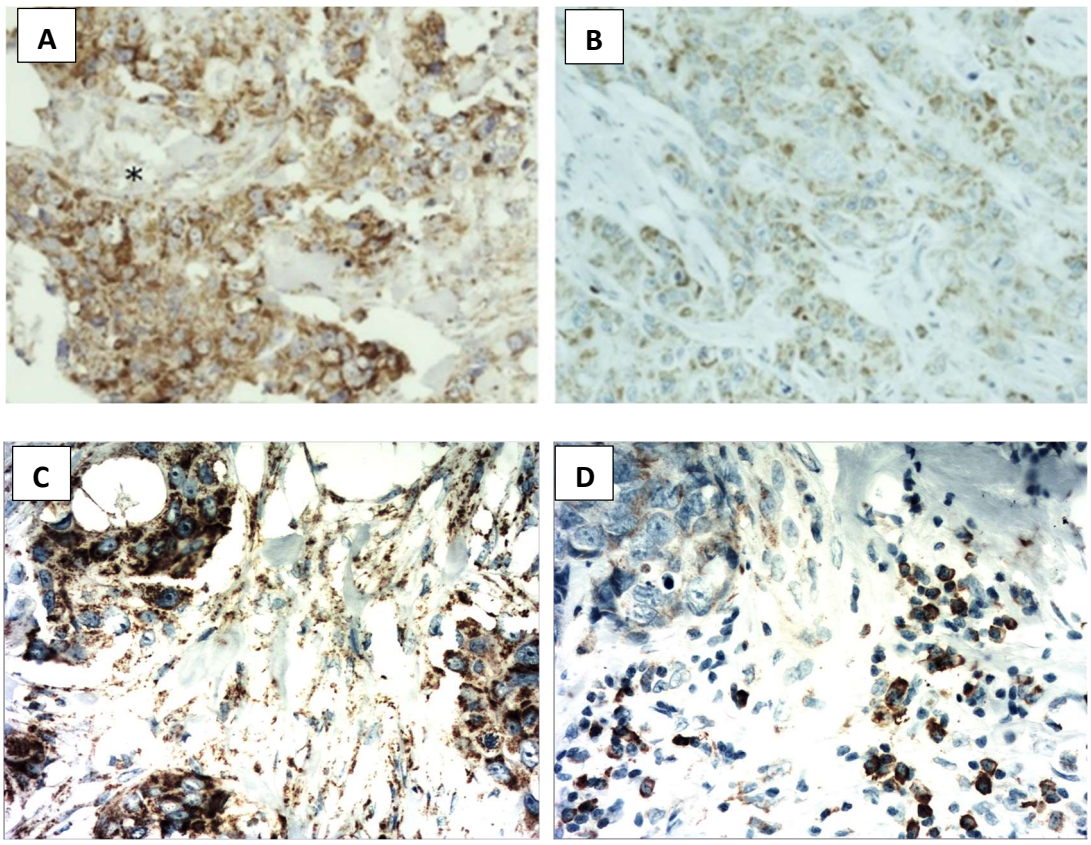
Parameters	N	%
Age (range 26-81 years)		
< 40	9	13.6
> 40	57	86.4
Histological sub-type		
invasive ca, NST	53	80.3
apocrine ca	8	12.1
adenoid cystic ca	2	3
adenosquamous ca	2	3
salivary like, NST	1	1.5
Tumor size		
pT1b	10	15.2
pT1c	27	41
pT2	28	42.4
pT3	1	1.5
Tumor grade		
G1	3	4.5
G2	11	16.7
G3	52	78.8
Lymph node status		
negative	49	74.2
positive	17	25.8
Recurrence		
present	18	27.3
absent	36	54.5
not available	12	18.2
Survival	66	
dead	12	18.2
dead from cancer	10	15.2
alive	42	63.6
not available	12	18.2

ca, cancer; NST, no special type

4.2. Analýza proteínu CD9 v nádorových a stromálnych bunkách

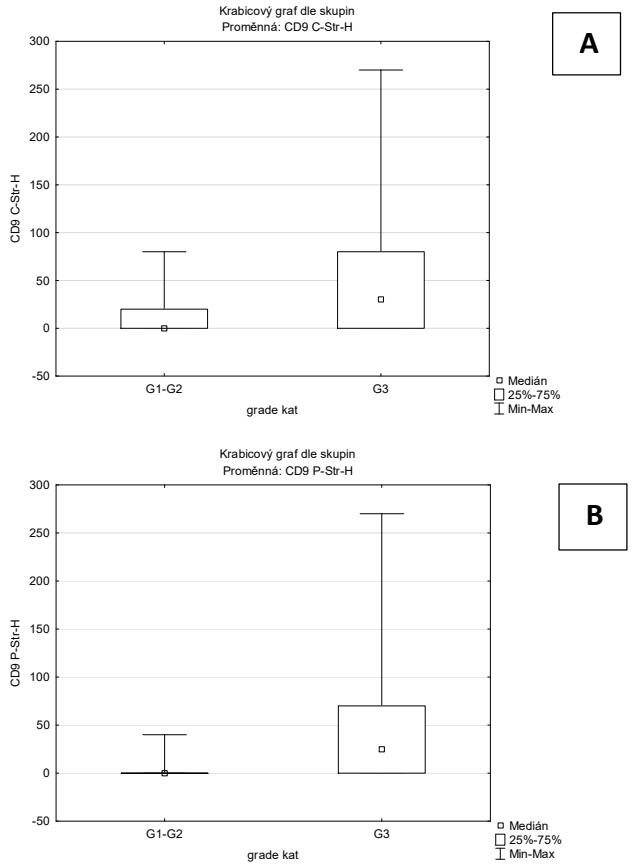
Cytoplazmatickú expresiu CD9 sme hodnotili v nádorových bunkách a v stróme pomocou H skóre s rovnakými stupňami intenzity ako pri CD29. Expresia CD9 v uzlinových metastázach bola signifikantne nižšia (p=0.013), v porovnaní s primárnymi karcinómami (Obr. 11.A-B), s grafickým zobrazením tejto skutočnosti Obr.13A).

Stromálna expresia CD9 sa týkala expresie najmä v imunitných bunkách.
U niektorých nádorov sme pozorovali extracelulárnu pozitivitu CD9 (Obr. 11 C-D), ktorá bola prítomná predovšetkým u nádorov s vyšším grade (16 z 17 nádorov). Tento typ expresie nebol spojený s uzlinovými metastázami, alebo s vyšším pT tumoru.

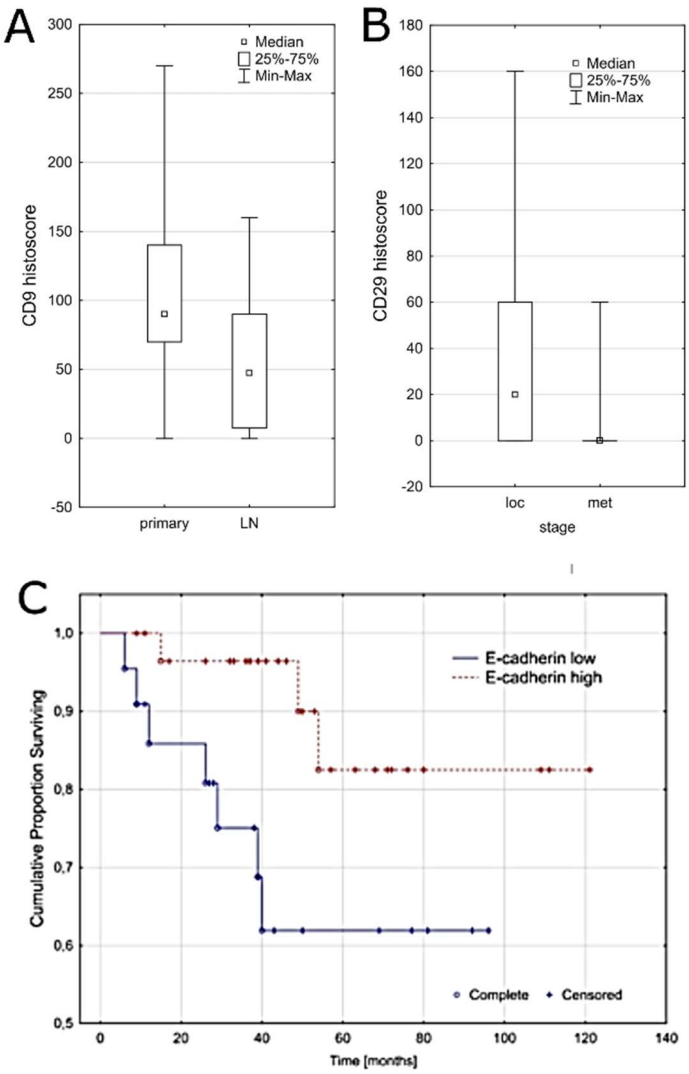


Obr. 11 Proteín CD9. (A)- vyššia expresia CD9 v primárnom tumore, v porovnaní s nízkou expresiou v MTS lymfatické uzliny (B). C - stromálna expresia v imunitných bunkách, D - zvláštna extracelulárna expresia intensity 2+.

Stromálna expresia CD9 na periférii (CD9 P-Str H) aj v centre nádora bola signifikantne spojená s vyšším histologickým grade tumoru (Obr.12).



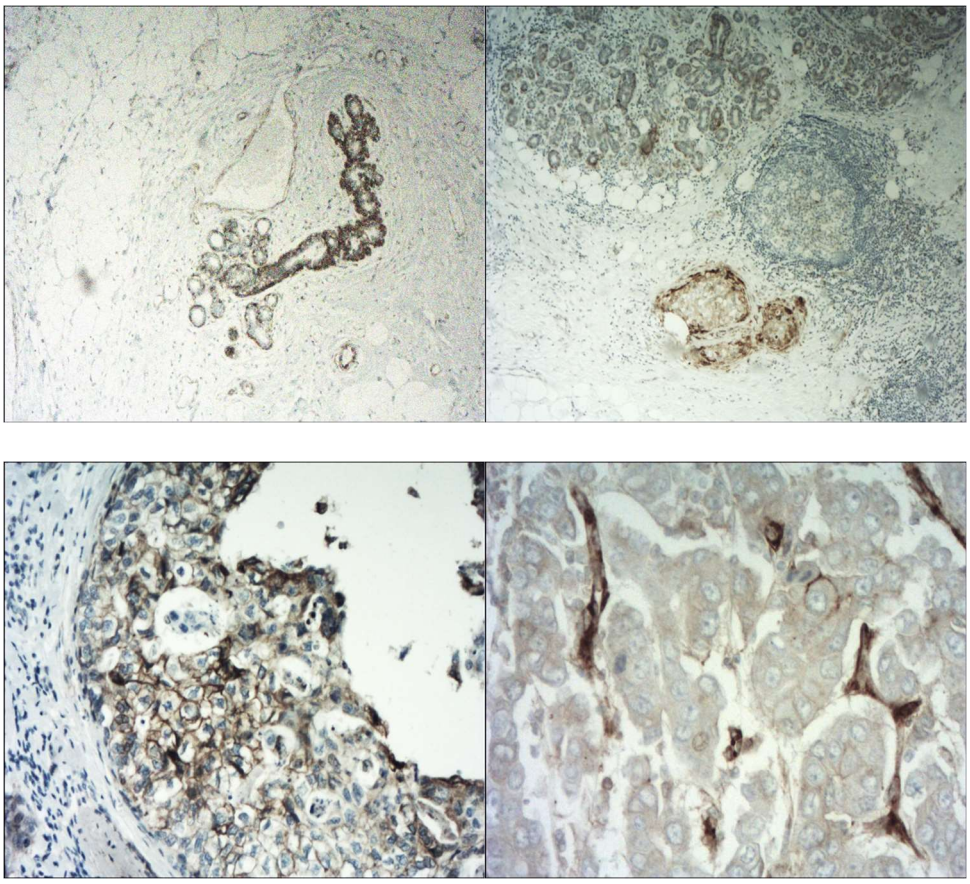
Obr. 12 Stromálna expresia CD9. (A) - Stromálna expresia CD9 v centre (CD9 -C-Str-H; p=0.045), (B) aj na periferii nádora (CD9 -P-Str-H, p=0.010) je signifikantne spojená s vyšším histologickým grade tumoru.



Obr. 13. Vzťah CD9, CD29 a E cadherínu k metastázam v lymfatických uzlinách a špecifickému prežitiu na mamárny karcinóm (BCSS). Expresia CD9 bola signifikantne vyššia v primárnych tumoroch v porovnaní s lymfatickými metastázami (A, p = 0.021). Expresia CD29 bola signifikantne vyššia u pacientov s lokalizovanou chorobou v porovnaní s tumormi s lymfatickými metastázami (B, p = 0.030). Nižšia expresia E cadherínu na periférii tumoru bola asociovaná s horším BCSS (C, p=0.038). Krabicové grafy predstavujú 25-75 percentil, medián a rozsah hodnôt.

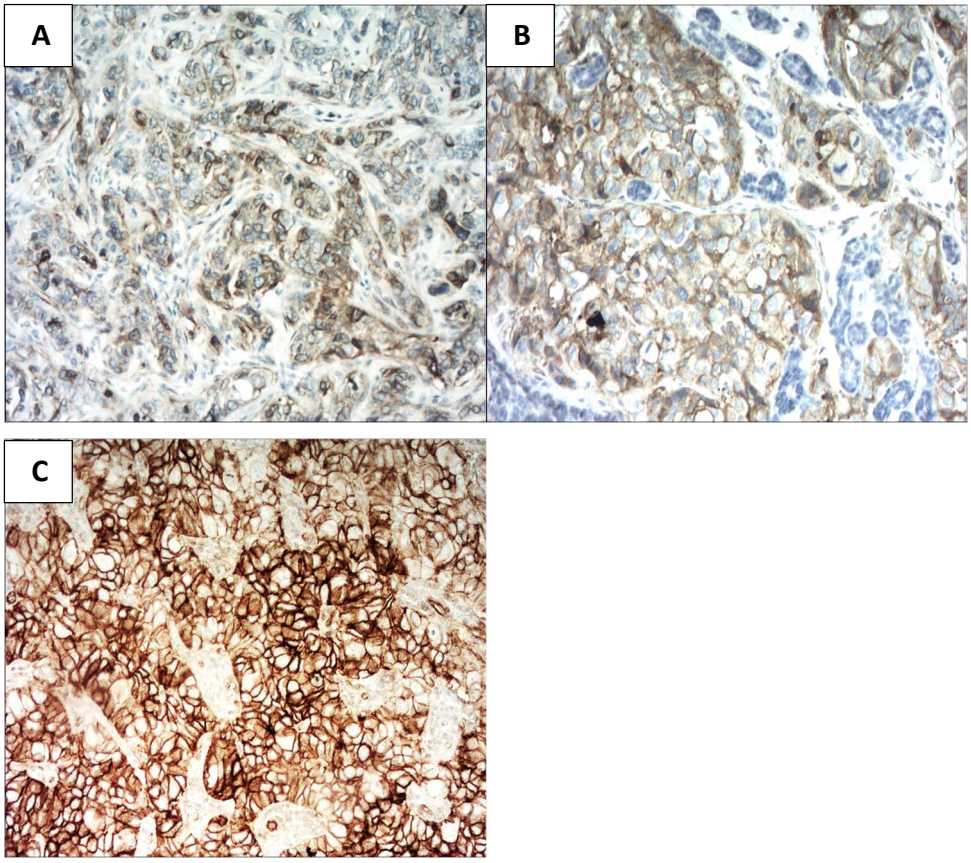
4.3. Hodnotenie exprese proteínu CD29 v nádorových bunkách

CD29 marker imunohistochemicky vykazoval cytoplazmatickú a membránovú expresiu. Marker bol slabo exprimovaný v duktulolobuloch normálnej prsnej žľazy, podobne ako v literatúre (Damjanovich a kol., 1997). Oblasti DCIS vykazovali silnejšiu expresia v porovnaní s normálnymi duktulolobulmi prsnej žľazy. Drobné krvné cievy v nádore tvorené endoteliami s pozitívnou expresiou slúžili ako vnútorná kontrola farbenia (Obr.14A-D)

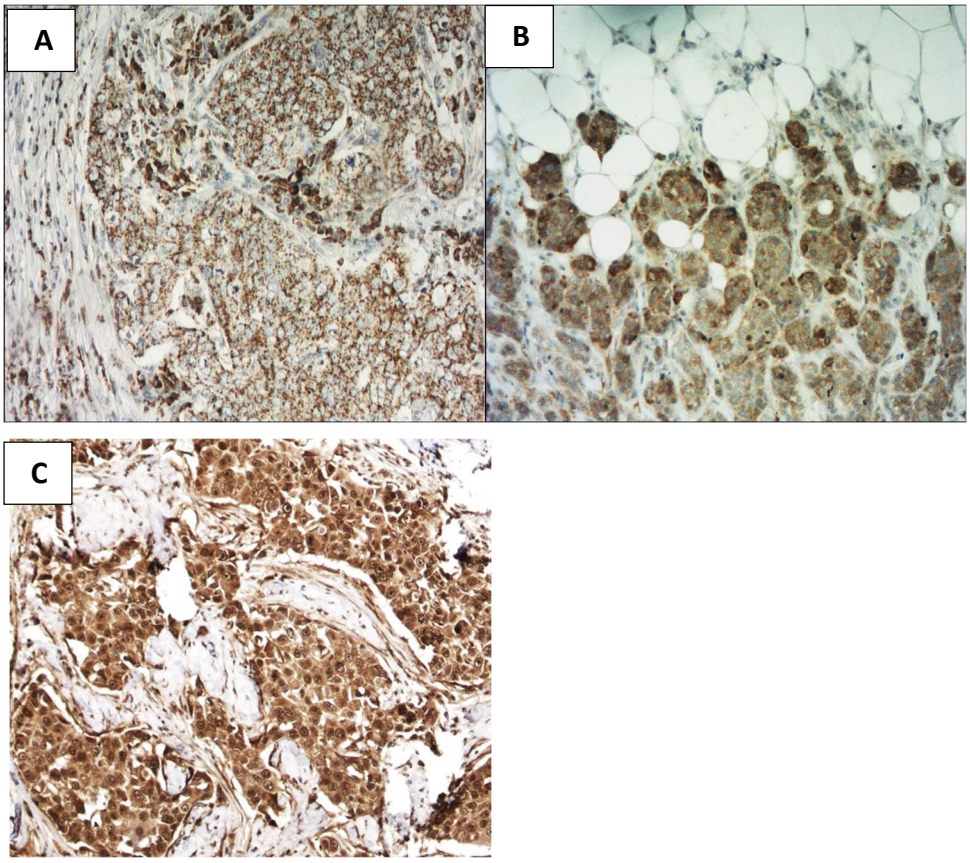


Obr. 14 Proteín CD29. A - expresia CD29 v normálnych duktulolobuloch prsnej žľazy slabšej intensity, B - silnejšia expresia CD29 v DCIS solidní typ, C- detail membránovej expresie CD29 intenzity 2+ v DCIS, D - CD29 v endoteliách nádorovej vaskulatury slúžiaca ako interná kontrola farbenia.

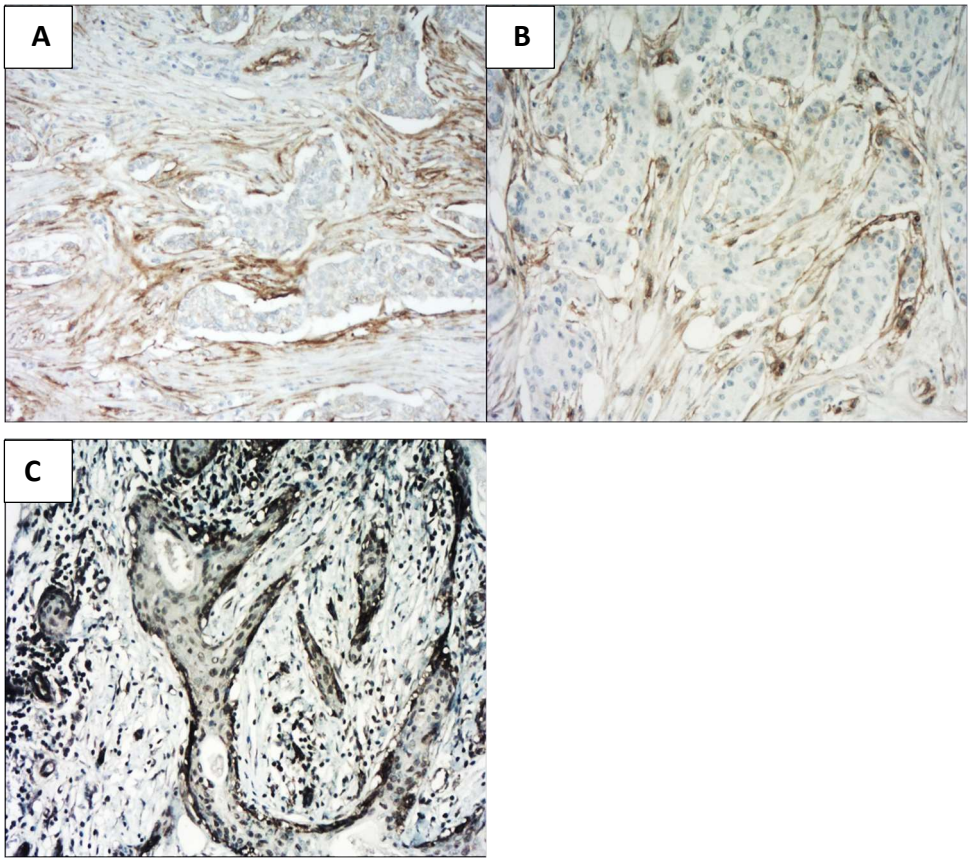
Povaha expresie niektorých nádorov bola prevažne membránová (Obr. 15A-C), ktorá je udávaná aj v recenzovaných publikáciách (Hsiang-Chun a kol., 2014). Niektoré nádory vykazovali dominantne cytoplazmatickú povahu expresie (Obr.16 A-C). Nádorová desmoplastická myofibroblastická stróma fokálne exprimovala CD29. Miestami sme pozorovali silnejšiu expresiu nádorových epitelových buniek v oblasti tzv. epitelo-väzivového spojenia (tzv. epitelo-mezenchýmovej junkcie) pripomínajúce štruktúry bazálnej membrány (Obr.17 A-C) popisovanej i v literatúre (Damjanovich a kol., 1997). Štatistická analýza ukázala, že expresia CD29 bola signifikantne vyššia u pacientiek s lokalizovanou chorobou v porovnaní s nádormi s lymfatickými mts (p= 0.030), (obr.13B), s imunohistochemickým obrazom expresie CD29 v oboch primárnych nádoroch popísaných vyššie (18 A-B). V ojedinelých nádoroch sme pozorovali osamotené nádorové bunky v tzv. prednej línii s inváziou do strómy so silnou expresií CD29 (obr 18C).



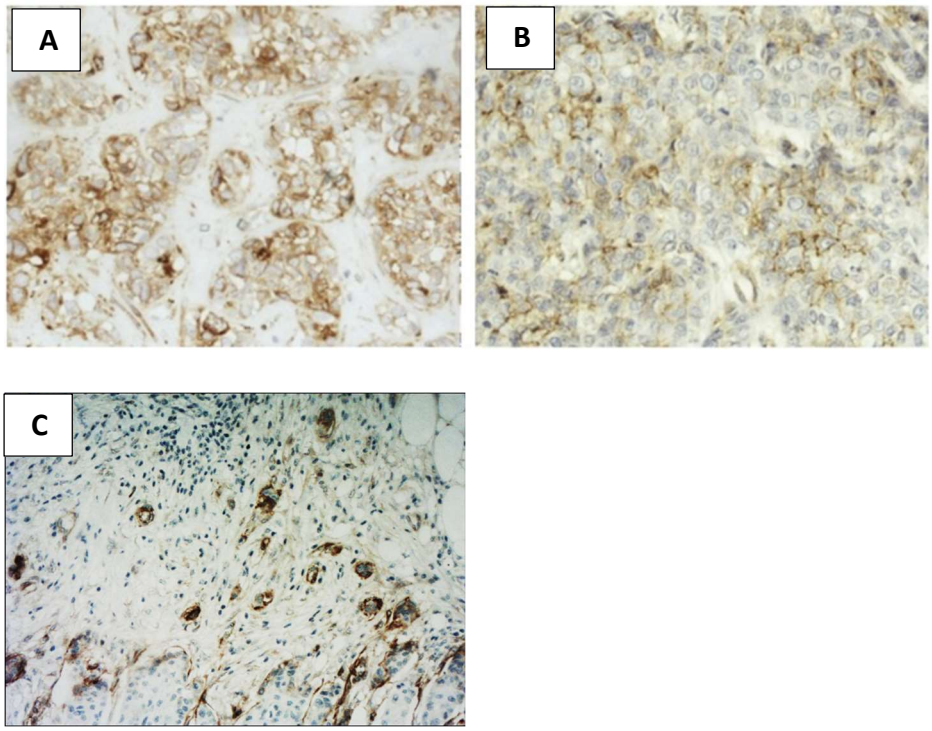
Obr. 15 Príklady prevažne membránovej expresie CD29. A - membránová expresia 1+, B - expresia membránová 2+, a cytoplazmatická 1+, C - membránová expresia 3+.



Obr. 16 Príklady prevažne cytoplazmatickej expresie CD29. A - cytoplazmatická expresia 1+ , B - cytoplazmatická expresia 2+, C - expresia cytoplazmatická 3+ a membránová 1+.



Obr. 17 Proteín CD29. A,B - pozitívna expresia myofibroblastickej nádorovej strómy, C - nápadná silná expresia v bazálnych častiach nádorového epitelu v oblasti epitelo-mezenchymálnej junkcie.

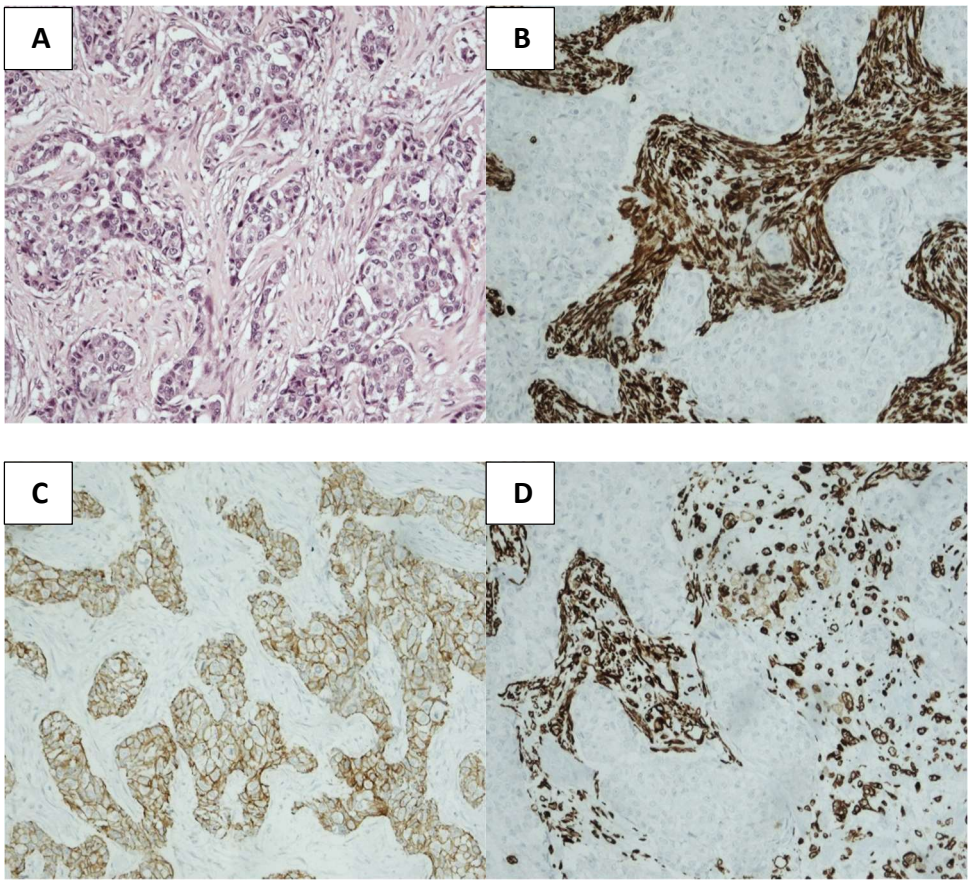


Obr. 18 Proteín CD29. A - silnejšia expresia v primárnom nádore s pTN0, B – slabá expresia v nádore s lymfatickými metastázami, C - silná expresia osamotených nádorových buniek v prednej línii na periférii nádoru, ktoré sú ukazovateľmi invazívneho infiltratívneho nádorového rastu.

4.4. Ďalšie sledované proteíny

Ďalšími parametrami, ktoré sme sledovali bola proliferačná aktivita nádoru a imunohistochemická expresia markerov, ktoré definujú epitelový a mezenchýmový fenotyp nádorovej bunky : E cadherín a vimentín. E cadherín, s membránovou expresiou, a vimentín, ako cytoskeletálny marker s membránovo-cytoplazmatickou expresiou, boli hodnotené pomocou H skóre s trojstupňovou intenzitou od 1+ až do 3 (slabej, strednej a silnej intenzity). **Vimentín** bol typicky exprimovaný v desmoplastickej nádorovej stróme, a v nádorových bunkách bol väčšinou negatívny. **Expresia E cadherínu** bola opačná s typickou epitelovou pozitivitou nádorových buniek a negativitou mezenchýmovej nádorovej strómy. V niektorých nádoroch bola prítomná fokálna pozitívna expresia vimentínu v neoplastických

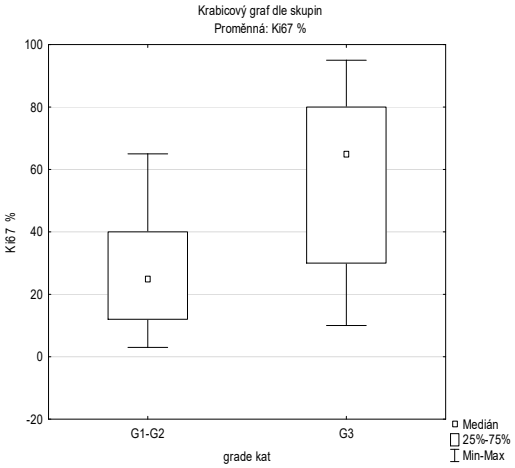
epitelových bunkách (Obr.19A-D). Tieto markery sme korelovali medzi sebou navzájom a hľadali medzi nimi súvislosti vzhľadom ku stavu tumoru (pT), grade tumoru, a prognóze.



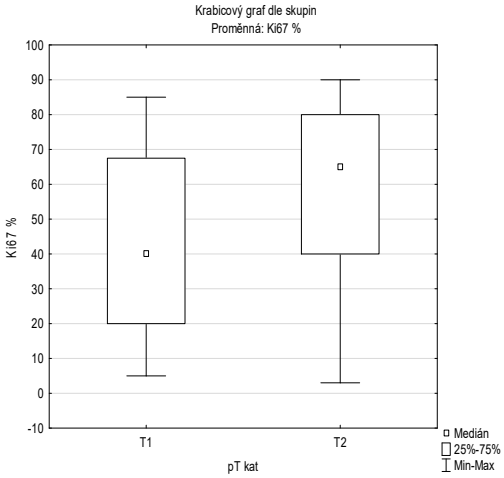
Obr. 19 Proteíny vimentin a E-cadherin. A - invazívny karcinóm (hematoxylin-eozín), B - pozitívna expresia vimentínu v desmoplastickej nádorovej stróme, a negativita v nádorových b. C - pozitívna expresia E cadherínu v nádorových b. D - prekvapivá fokálna pozitívna expresia vimentínu v nádorových b.

Nízka expresia E cadherínu na periférii primárneho tumoru bola spojená s horším nádorovo špecifickým prežívaním (p=0.03) (Obr.13C). Proliferačný index Ki67 taktiež signifikantne súvisí s vyšším grade i štádiom nemoci (obr.20). Vyššie Ki67 tiež koreluje s nižším vekom (Rs -0.32).

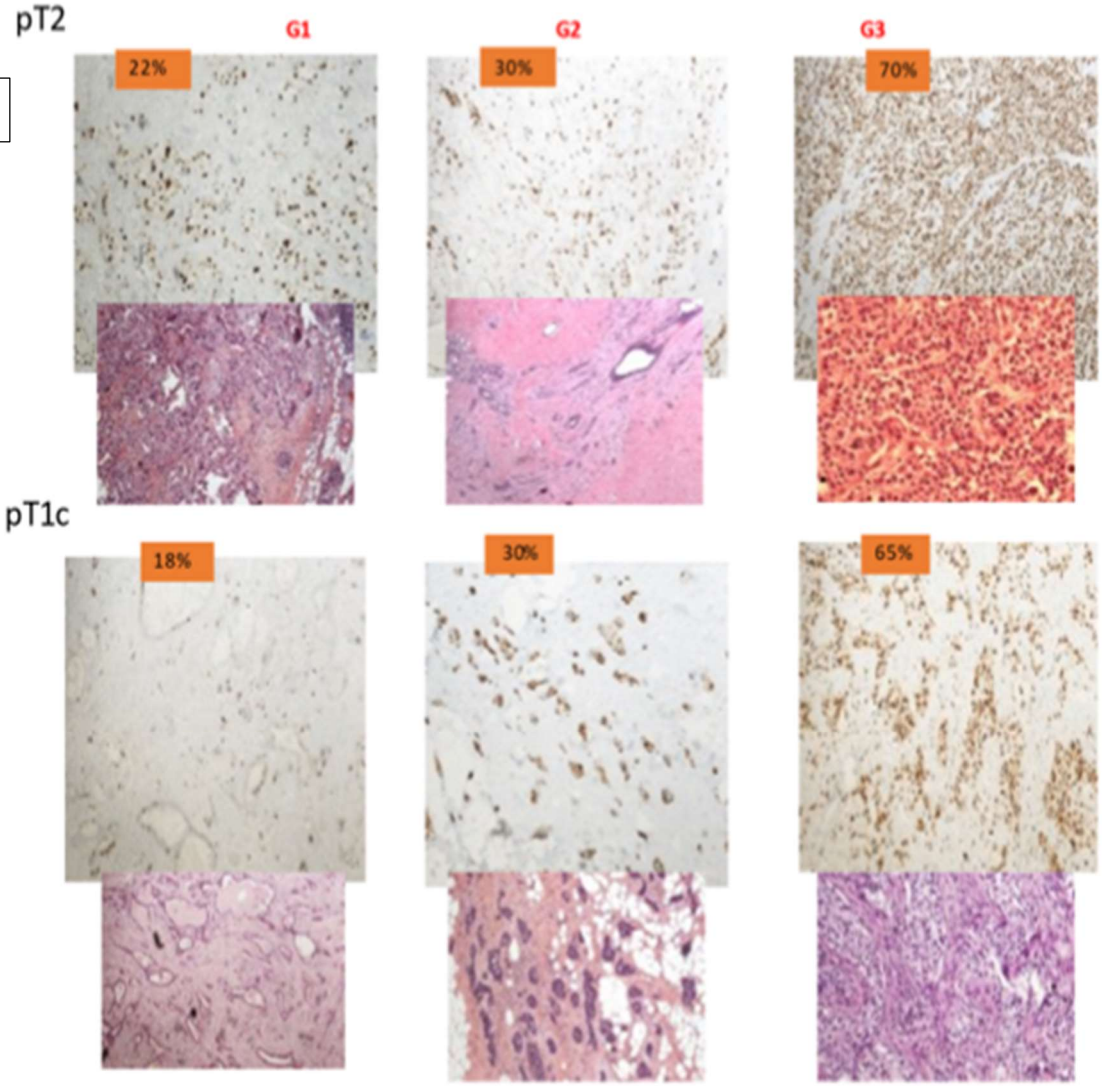
A


Krabicový graf dle skupin
Proměnná: Ki67 %
Ki67 %
100
80
60
40
20
0
-20
G1-G2
G3
grade kat
Medián
25%-75%
Min-Max

B

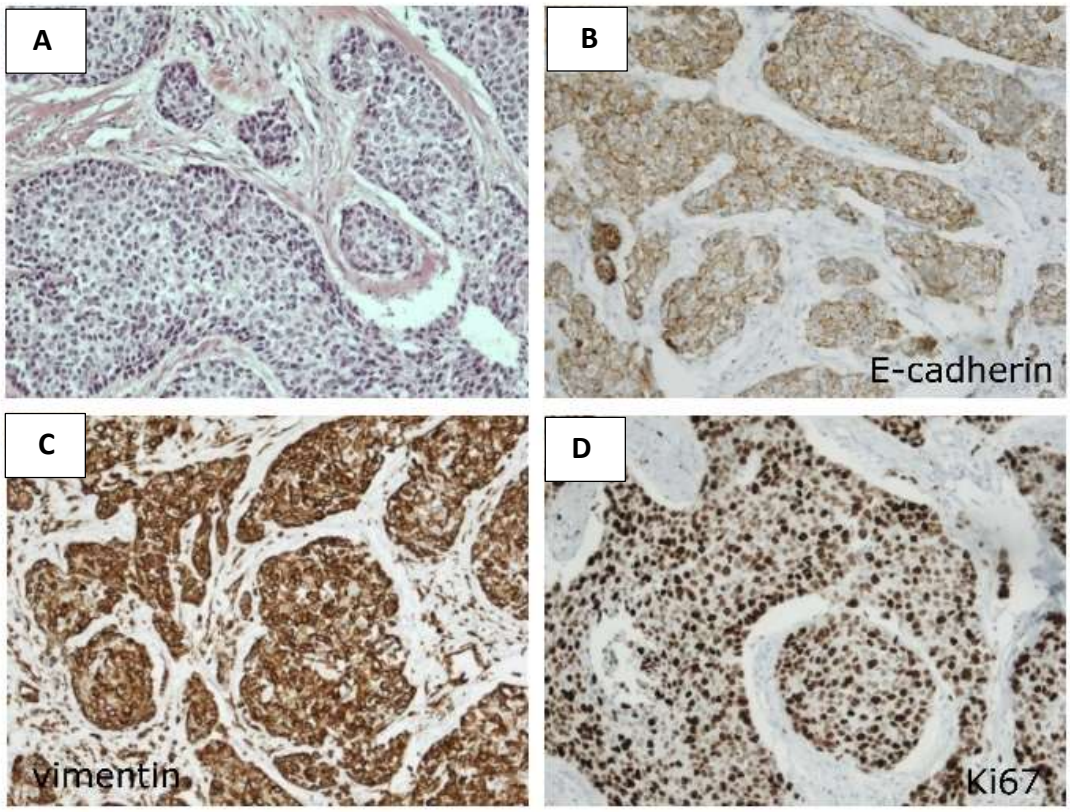

Krabicový graf dle skupin
Proměnná: Ki67 %
Ki67 %
100
90
80
70
60
50
40
30
20
10
0
-10
T1
T2
pT kat
Medián
25%-75%
Min-Max

C


pT2
G1
G2
G3
22%
30%
70%
pT1c
18%
30%
65%

Obr. 20 Vzťah Ki67 s vyšším grade i štádiom nemoci. Proliferačný index Ki67 koreluje s vyšším histologickým grade (A) i štádiom nemoci-pT (B). Čím vyšší grade a pT choroby tým vyššia proliferačná aktivita karcinómu, proliferačný index koreluje s vyšším pT, s ilustráciou spomínaných asociacií u vybraných nádorov (C).

Dôležitým objavom bol fakt, že nízka expresia E cadherínu na periférii bola spojená a vyššou expresiou vimentínu (Rs-0.33) a s vyšším Ki 67 (Rs-0.26) (Obr.21).

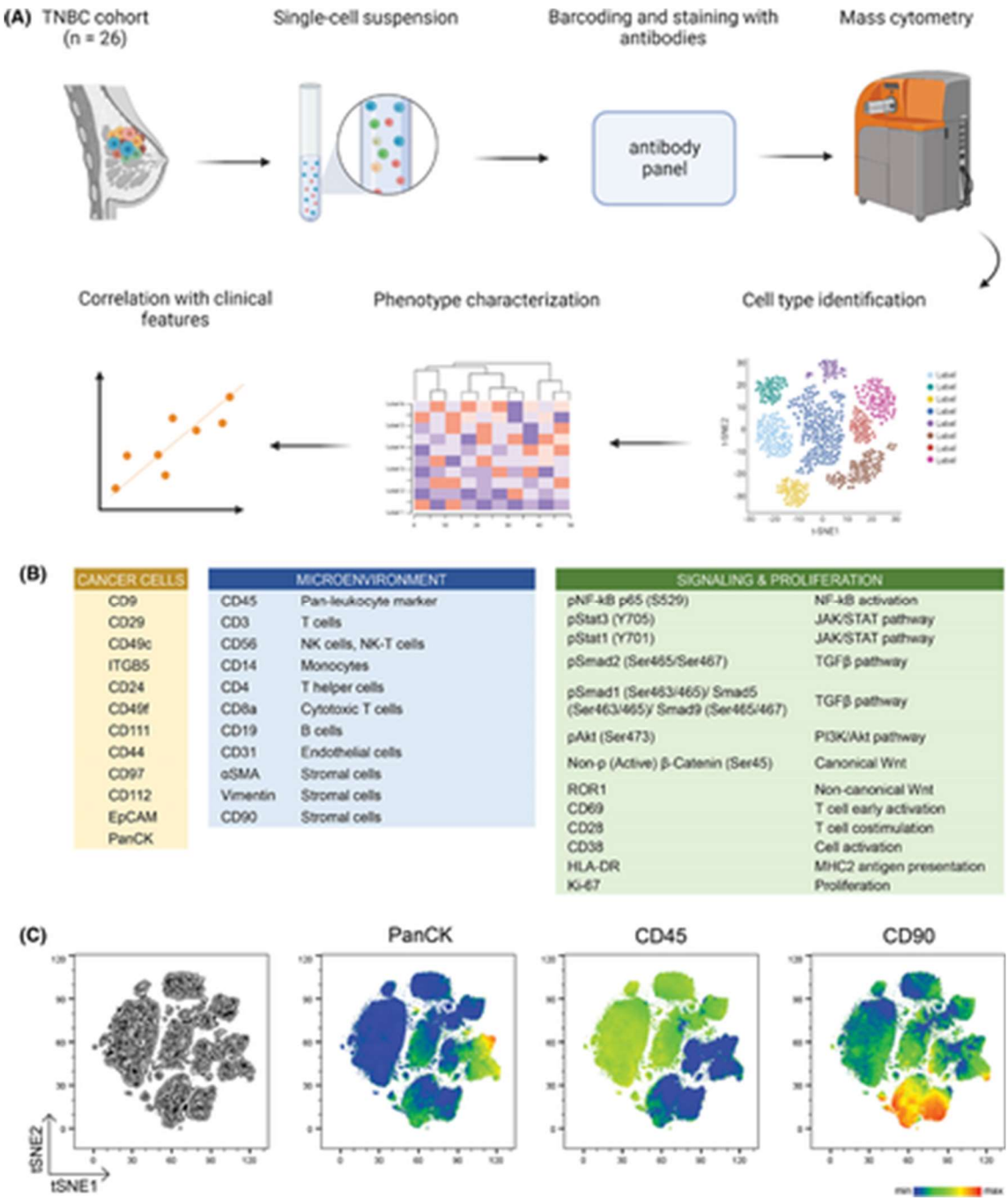


Obr. 21 Exprese E-cadherinu, vimentinu a Ki67 v rovnakom ložisku u vybraného prípadu TNBC. A - TNBC (hematoxylin -eozín), B - nízka expresia E cadherínu na periférii bola spojená s vyššou expresiou vimentínu (C), a s vyšším Ki 67 (D).

4.5. Zvýšená expresia CD97 je spojená s horším celkovým prežitím

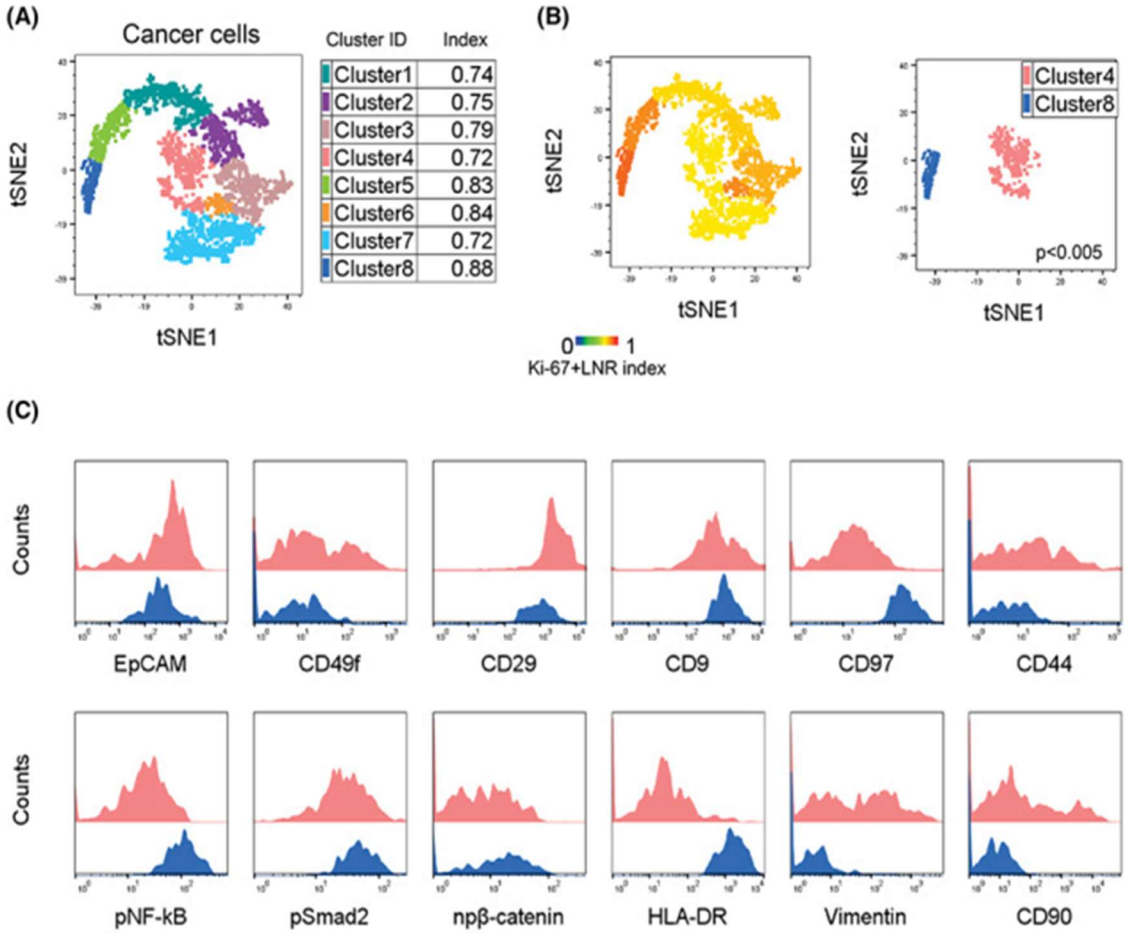
Okrem vyššie uvedenej štúdii na kohorte pacientiek diagnostikovaných v Ostravě, sme prispeli aj k analýze súboru z Masarykova onkologického ústavu v Brně (Kvokačková et al. 2023). Cielom práce bolo pomocou 36 markerov klasifikovať epitelo-mezenchymálnu plasticitu nádorových buniek TNBC, a molekulárne definovať fenotypicky rôznorodú populáciu nádoru a tumor asociovanej strómy.

Resekovaný materiál bol enzymaticky disociovaný na jednotlivé bunky, ktoré boli ofarbené panelom protilátok a analyzované pomocou hmotnostnej cytometrie (obr. 22A). Panel protilátok bol navrhnutý, aby identifikoval subpopulácie nádorových buniek, o ktorých je známe, že prispievajú k progresii nádorov prsníka, povrchové molekuly, ktoré sa spájajú s plasticitou EMT/MET (napr. EpCAM a Vimentin) a súbor nových povrchových antigénov odrážajúcich plasticitu buniek nádorov (napr. CD29, CD97, CD49c, ITGB5). Boli tu zahrnuté aj markery, ktoré umožnili podtypovanie stromálnych buniek (napr. CD90, Vimentin a alfa SMA), imunitných buniek (napr. CD45, CD3, CD14, CD19), a analýzu vybraných signálnych dráh (TGF-β/SMAD, NF-κB, JAK-STAT, PI3K/AKT/mTOR a Wnt) (obr. 22B). Väčšina buniek bola hematopoetického pôvodu (CD45+), potom nasledovali stromálne (CD90+) a nádorové kompartmenty (PanCK+). Všetky tieto markery boli vizualizované farebne pomocou t-SNE máp (obr. 22C).



Obr. 22 Charakteristika heterogenity nádorov vo vzorkách TNBC pomocou hmotnostnej cytometrie. (A) Schéma zobrazujúca experimentálny a analytický pracovný postup pre primárne vzorky pacientov s TNBC použité v štúdii Kvokačková a kol. (2023). (B) Zoznam bunových povrchových a signálnych molekúl vybraných pre charakterizáciu kompartmentu nádora a nádorového mikroprostredia. (C) Dvojrozmerná t-SNE vizuálizácia expresie pan CK, CD45 a CD90 vo všetkých buňkách a vzorkách (n=26). Kombinácia týchto troch markerov bola použitá pre identifikáciu nádorových (Pan CK+), imunitních (CD45+) a stromálnych (Pan CK-CD90+) buniek.

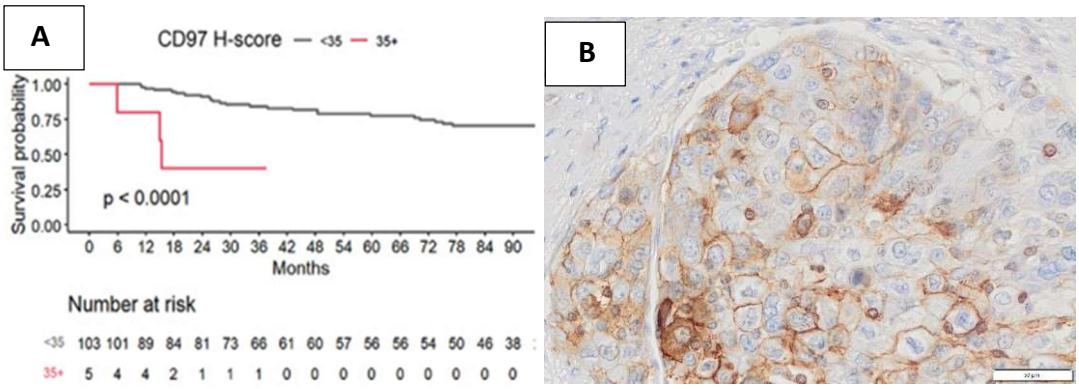
Pretože pre analyzované pacientky zatiaľ neboli informáce o dlhodobom prežívaní, tak bol ako parameter agresivity ochorenia vytvorený index Ki-67 + LNR, ktorý sa skladal z klinického hodnotení metastáz do uzlín a pozitivity Ki-67. Spolupracovníci identifikovali osem fenotypovo odlišných populácií buniek PanCK+ (obr.23A).



Obr. 23 Komplexná analýza nádorových buniek v tumoroch TNBC. (A) t-SNE graf ukazujúci zhluky nádorových buniek ofarbených pomocou FlowSOM. (B) Graf t-SNE ofarbený indexom Ki 67+LNR (vľavo), s vyobrazenými populáciami buniek Ki 67+LNR -high Cluster 8 (modrá) a low Cluster 4 (ružová) v pravom panele. (C) Histogramy zdorazňujú rozdiel v expresii vybraných proteínov v Ki 67+LNR -high Cluster 8 (modrá) a low Cluster 4 (ružová).

Tieto populácie mali odlišné expresné vzorce EMT/MET markeru vimentin, radu bunkových adhéznych molekúl a integrinov (CD29, CD49f, CD97, CD44, CD90) a signálnych molekúl (pNF-κB a HLA-DR) (viz Kvokačková a kol., 2023). Tieto subpopulácie TNBC sa líšili v svojich indexoch Ki-67 + LNR. Cluster 8 mal najvyšší vypočítaný index Ki-67 + LNR (modrý; 0,88) a cluster 4 mal najnižší (ružový; 0,72) (obr. 23B). Histogramy ukazujú rozdiel v expresii vybraných proteínov. Nádorové bunky prítomné v clusteru 8 vykazovali zvýšenú expresi CD97, pNF-KB a HLA-DR (obr. 23C). Expresia týchto proteínov (spoločne s ITGA3 / CD49c, ktorý bol vybraný na základe analýzy stromálnych buniek (viz Kvokačková a kol., 2023) boli sledované pomocou imunohistochémie na nezávislom súbore 117 TNBC pacientiek (TMA z primárnych nádorov), u ktorých bol k dispozícii dlhodobý follow-up.

Výsledky ukázali, že iba zvýšená expresia CD97 nádorovými bunkami bola významne spojená s horším celkovým prežitím (obr. 24).



Obr. 24 C97 proteín. A - Kaplan-Meierova krivka ukazujúca celkové prežitie pacientov s TNBC na základe expresie CD97 v nádorových buňkách (n=108, log rank test). B - pozitívna imunohistochemická expresia C97 z TMA preparátu.

5.Diskusia

Naše výsledky o nižšej expresii CD9 v uzlinových metastázach sú v zhode s niektorými prácami o solídnych nádoroch v literatúre. Týto autori zistili, že čím vyššia expresia CD9 v nádore tým je nádor menej agresívny, má menšie riziko metastázovania a má lepšiu prognózu (Arihiro a kol., 1998, Houle a kol., 2002, Sauer a kol., 2003, Mhawech a kol., 2003, Hashida a kol., 2003, Amatya a kol., 2013, Miyamato a kol., 2001, Wang a kol., 2007). Týkalo sa to kolorektálnych, mamárnych, prostatických, endometriálnych, cervikálnych, pľúcnych karcinómov a mezoteliómu. Avšak niektorí autori zistili opačný význam CD9 v zmysle zvýšenej expresie CD9 v agresívnejších nádoroch. Tento poznatok zistili u melanomu, karcinomov žaludka, štítnej žlazy, pankreatických adenokarcinomov a klinicky metastatických ovariálnych karcinómov s lymfogénnou inváziou (Kim a kol., 2019, Hori a kol., 2004, Soyuer a kol., 2010, Lucarini a kol., 2022, Hwang a kol., 2012). Keď sa sústredíme na mamárne karcinómy vatšina prác spája zvýšenú expresiu CD9 s lepšou prognozou nádorov, avšak práca autorov o lobulárnom karcinome a v type luminal A, NST typu karcinómu zistila, že zvýšený CD9 v tomto type nádorov je spojený s horšou prognózou (Baek a kol., 2019, Kwon a kol., 2017). Podobný fakt o zlej prognóze nádorov so zvýšenou expresiou CD9 zistili autori publikácie o kostných metastázach mamarného karcinomu, v porovnaní s expresiou v primárnych nádoroch prsnej žlazy (Kischel a kol., 2012).

CD9 podľa niektorých prác znižuje motilitu buniek a pomáha pri adherencii (priľnavosti) buniek k okolitej stróme (Furuya a kol., 2005). Naše výsledky štúdie, že nízka expresia CD9 a CD29 je asociovaná s nádormi s lymfatickými

metastázami sú do určitej miery v zhode s pozorovaním, že znížená expresia CD9 a CD29 v mamárnych karcinómoch je spojená s nádorovými bunkami, ktoré podliehajú procesu EMT (Remšík a kol., 2018). Ale nepozorovali sme koreláciu CD9 a CD29 s E cadherínom a vimentínom, ani nádorovo špecifickým prežívaním.

Teóriu o význame adhézie buniek a molekule CD9 detailnejšie rozvinula štúdia (Germana a kol., 2015), ktorá skúmala morfológiu rôznych výbežkov membrány nádorových buniek in vitro a zistila, že zvýšená expresia CD9 je potrebná k tvorbe špecifických výbežkov plazmatickej membrány nádorovej bunky, ktorá im pomáha v procese invázie do strómy. V tomto zmysle zvýšenie CD9 pozitívnych plazmatických výbežkov podporovalo a uľahčovalo invazivitu nádoru. Zvýšené CD9 zvyšuje kapacitu nádorových buniek k invazivite a metastázovaniu a môže byť znakom lymfogénnej propagácie niektorých nádorov ako je melanom, karcinom ovária a hepatocelulárny karcinom (Soyuer a kol.,2010, Qing a kol., 2018, Lucarini a kol., 2022). CD9 uľahčuje lymfogénnu propagáciu zhubných nádorov tým, že podporuje vaskulárne rastové faktory a podieľa sa na aktívnej redistribúcii endoteliálnej CD9 u niektorých nádorov počas interakcie nádorová bunka a endoteliálna bunka vo fázy intravazácie pri procese metastázovania zhubných nádorov (melanom, Longo a kol., 2001).

Niektorí autori si všímali stromálnu expresiu CD9 v imunitných bunkách a zistili, že tento typ expresie CD9 je spojený s lepšou prognózou u nádorov prsnej žlazy (Kwon a kol., 2017) a horšou prognózou u nádorov hrubého čreva (Kim a kol., 2016). V našej práci sme pozorovali asociáciu stromálnej expresie CD9 a pacientiek s vyšším grade. Stromálna expresia CD9 sa týkala expresie najmä v imunitných bunkách, vrátanie tumor infiltrujúcich lymfocytov (TILs). Vzťah TILs a vyššieho grade už bola pre TNBC popísaná

(Tian a kol. 2016, Kolečková a kol. 2019).

Pôvod zvláštnej extracelulárnej stromálnej pozitivity CD9 nie je celkom jasný. Vysvetlením môže byť publikácia Yuichiro Miki a spol., ktorí študovali extracelulárnu pozitivitu CD9 v in vitro podmienkach, kde táto pozitivita reprezentuje exozómy tzv. „cancer associated“ fibroblastov, ktoré sú zodpovedné za intercelulárnu komunikáciu v nádore. V tejto práci zistili, že tieto exozómy signifikantne stimulujú inváziu a migráciu nádorových buniek v karcinómoch žalúdka skirhotického typu (Miki a kol.,2018). Táto hypotéza podporuje fakt, že tento typ expresie CD9 môže byť spojený s agresívnejším správaním nádorov. Fibroblasty sú súčasťou každej desmoplastickej strómy nádora a tvorí tzv. stromálne mikroprostredie nádora. Ďalšími možnosťami pokročiť v týchto analýzach môže byť využitie iných farbení (TUNEL pre apoptózu), alebo sledovaním iných znakov pre exozómy/mikrovezikuly.

Niektoré štúdie potvrdzujú fakt, že CD29 expresia môže byť morfologickým markerom EMT (Songmei a kol., 2017). Autori zistili, že bunky s fenotypom CD29+/CD44+ so silnou expresiou týchto markerov mali vyššiu proliferačnú aktivitu a lepšiu schopnosť invázie a metastázovania. Iné práce ukazujú opačný fakt, že chýbanie markera CD29, spolu s CD9, ako markera epitelového fenotypu, charakterizujú nádorové bunky, ktoré sú zapojené do EMT (Remšík a kol., 2018). Autori niektorých publikácii (Vassilopoulos a kol., 2014, Lisiak a kol., 2017, Songmei a kol., 2017) zistili, že CD29 pozitívne nádorové bunky tkanivových kultúr zvyšujú migračnú aktivitu a invazivitu týchto buniek, a tým vlastné metastázovanie tumoru. Z toho vyplýva, že CD29 je možným prognostickým ukazovateľom, v zmysle, že silnejšia expresia znamená horšiu prognózu tumoru (Vassilopoulos a kol., 2014, Lisiak a kol., 2017) a horšie prežívanie TNBC (Klahan a kol., 2016, Jin a kol., 2016, Pleiko a kol., 2023). Iná práca zistila podobný záver s našou, že

chýbanie CD29 v bunkách oslabuje rast mamárnych karcinómov, ale značne napomáha diseminácii nádorových buniek do pľúc (Truong a kol., 2014). Náš výsledok o CD29, ukázal veľmi podobnú skutočnosť, že membránová expresia CD29 bola signifikantne nižšia v primárnych nádoroch s uzlinovými metastázami ako v nádoroch bez metastáz. Pozorovanie osamotených nádorových buniek so silnou expresiou CD29 v tzv. prednej línii s inváziou do strómy možno vysvetliť funkciou CD29 ako integrínu, ktorý interaguje s kolagénmi, a tieto interakcie pomáhajú pri invázii tumoru (Songmei a kol., 2017). V zhode s touto prácou sme tento jav silnej expresie CD29 v osamotených nádorových bunkách s infiltratívnym rastom na periférii tumoru pozorovali. Konfliktné výsledky o CD29 môžu byť spôsobené použitím detekcie CD29 v rôznych in vitro a in vivo podmienkach, alebo použitím súborov pacientov s malým počtom pacientov. Povaha imunohistochemickej expresie CD29 (cytoplazmatická alebo membránová), môže byť vysvetlená štruktúrou CD29 proteínu, ktorý má extracelulárnu membránovú i intracelulárnu cytoplazmatickú doménu.

Významným markerom EMT je znížená expresie E cadherínu, ktorá je spojená s horším prežívaním mamárnych karcinómov v našej práci. Niektoré štúdie opisujú rovnaký fakt, že horšie prežívanie TNBC pacientov je spojené s nízkou expresiou E cadherínu (Zhang a kol., 2018, Tang a kol., 2010, Arihiro a kol., 1998). Predovšetkým Zhang a kol. zistili, že nízky E cadherín znamenal signifikantne vyššie riziko agresívnosti TNBC, zahŕňajúc rekurenciu nádoru a metastázy do lymfatických uzlín (Zhang a kol., 2018).

Marker CD97, ktorý bol identifikovaný spolupracovníkmi (Kvokačková a kol., 2023) je kódovaný génom *ADGRE5* (Haman a kol., 1995; Adhesion G Protein-Coupled Receptor E5). Patrí do tzv. rodiny „ adhesion G protein-coupled receptor“ (Langenhan a kol., 2013). Má významné funkcie

v imunitnom systéme, reguluje aktiváciu myeloidných a lymfoidných buniek. Zohráva dôležitú úlohu v aktivácii T buniek a tvorbe cytokínov (Capasso a kol., 2006). Mimo imunitný systém hrá dôležitú úlohu v interakciách medzi bunkami navzájom. Proteín je exprimovaný vo veľkom počte rôznych typov solidných nádorov. Výsledok našej publikácie Kvokačková a kol., že zvýšená expresia CD97 v TNBC je spojená s horšou prognózou je v zhode s vätšinou publikácií (He a kol., 2015, Wu a kol., 2012, Mustafa a kol., 2005). Niektoré práce jeho expresiu spájajú s inváziou nádorových buniek a s jeho prítomnosťou v prednej línii nádorových buniek, ktoré infiltrujú z periférie nádora do strómy pri progresii nádora (Wobus a kol., 2006, Safaee a kol., 2013).

6.Súhrn

TNBC sú heterogénna skupina nádorov väčšinou s agresívnym správaním u recidivujúcich tumorov s následným horším dlhodobým prežívaním. Jedným z faktorov, ktoré sú zodpovedné za inváziu, tvorbu metastáz, horšiu prognózu a rezistenciu týchto nádorov na chemoterapiu je jav EMT (epitelo-mezenchymová tranzícia).

Primárnym cieľom dizertácie bolo sledovať kvalitatívne a kvantitatívne imunohistochemickú expresiu proteínov CD9 a CD29 vo všetkých zložkách nádoru. Tiež sme tieto proteiny vyšetrovali v lymfatických uzlinách s metastázami, a porovnávali intenzitu expresie v primárnom nádorei v metastázach. Ďalej sme sledovali expresiu epitelových a mezenchymálnych markerov E cadherínu, a vimentínu, dôležitých pre definíciu javu EMT nádora.

Tieto údaje sme vyhodnotili štatisticky, s ďalšími charakteristikami nádora, a zistili sme signifikantné zníženie expresie CD9 v uzlinových metastázach v porovnaní s primárnym nádorom. Pozorovali sme silnú asociáciu vysokej proliferačnej aktivity pomocou proliferačného markera Ki 67 s vyšším grade nádoru. Dominantne membránová expresia CD29 bola signifikantne nižšia v nádoroch s lymfatickými metastázami v porovnaní s lokalizovanými tumormi pTN0. Znížená expresia E-cadherinu na periférii primárnych nádorov korelovala s horším prežitím pacientiek. Zistili sme taktiež asociáciu medzi E-cadherinom, vimentinom a proliferačným markerom Ki67. Znížená expresia CD9 a CD29 bola spojená s tvorbou lymfatických metastáz, avšak ich asociácia s EMT a prežívaním nebola dokázaná.

V nezávislom súbore z Masarykova onkologického ústavu v Brně, kde spolupracovníci študovali tzv. plasticitu nádorových buniek pomocou epiteliálnych a mezenchymálnych markerov, sme zistili, že zvýšená expresia CD97 je významne spojená s horším celkovým prežitím.

7. Summary

TBNC represent a heterogeneous group of malignant tumors showing usually an aggressive behavior and also a worse survival in the recurrent tumours. EMT (epithelial to mesenchymal transition) appears to be one of the factors responsible for tumour invasiveness, metastases, worse prognosis and resistance to chemotherapy.

The primary aim of the theses was to evaluate qualitatively and quantitatively immunohistochemical expression of proteins CD9 and CD29 in all elements of tumor. The above mentioned proteins were also assessed in lymph nodes containing metastases and the level of expression of both studied proteins in primary tumours and in metastases was compared. Additionally, the expression of epithelial and mesenchymal markers E cadherin and vimentin was evaluated to establish their importance in EMT.

The statistical analysis found a significantly decreased expression of CD9 in lymphatic metastases in comparison with the primary tumors. Also, an increased expression of proliferation marker Ki67 was associated with higher grade of the primary tumors. Furthermore, the membranous positivity of CD29 in primary tumor was significantly lower in patients with lymph node metastases compared to patients without cancer dissemination. Lower expression of E cadherin at the periphery of the primary tumor was associated with worse breast cancer specific survival. We have also found association between E cadherin, vimentin and Ki 67 proliferation marker. Decreased expression of CD9 and CD29 was related to development of lymphatic metastases, however their association with EMT and survival has not been proven.

In an independent study on a TNBC cohort from Masaryk Oncological Department in Brno, assessing so called plasticity of malignant cells by epithelial and mesenchymal markers, we showed that the increased expression of CD97 was significantly associated with worse overall survival.

8. Zoznam skratiek

ADAM17	A Disintegrin And Metalloproteinase 17
alfa SMA	alfa hladkosvalový aktín
BC	breast cancer
BCSS	breast cancer specific survival
BLIA	„basal-like immune-activated typ
BLIS	basal like immune-suppressed typ
ECM	extracelulárna matrix
EMT	epitelo-mezenchymálna tranzícia
ESCRT	endosomal sorting complexes required for transport
EVs	extracelulárne vezikuly
gén *ADGRE5*	*gén kodujúci* Adhesion G Protein-Coupled Receptor E5
H score	histo score
IM	„ immunomudulatory“ typ
inhibítory PARP	inhibitory poly ADP-ripose) polymerázy
LAR	luminal androgén receptor
MES	mesenchymal typ
MDSC	myeloid-derivované supresorové bunky
MET	mezenchýmovo-epiteliálna tranzícia
MMP	metaloproteinázy
MSL	„mesenchymal stem -like“ typ
mts	metastázy
pEMT	parcialna epitelo-mezenchymálna tranzícia
PMN	premetastatická nika
TDE	“tumor derived“exozómy
TIL	tumor infiltrujúce lymfocyty
TAMs	„ tumor-associated“ makrofágy
TAN	„tumor-associated“ neutrofily
TNBC	triple negatívne karcinómy
UNS typ	unstable cluster

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11. Přílohy

1. **Ondruššek R.**, Brychtová S., Bezděková M., Bouchalová K., Vávrová Z., Souček K., Bouchal J. Immunohistochemical analysis of CD9, CD29 and epithelial to mesenchymal transition in triple-negative breast cancer. Klin Onkol (2024). doi: 10.48095/ccko20241, v tisku.

2. **Ondruššek R**., Kvokačkova B., Kryštofova K., Brychtova S., Souček K., Bouchal J. Prognostic value and multifaceted roles of tetraspanin CD9 in cancer. Front. Oncol. (2023); 13: 1140738. doi: 10.3389/fonc.2023.1140738, IF: 6.244.

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Immunohistochemical analysis of CD9, CD29 and epithelial to mesenchymal transition in triple-negative breast cancer

Imunohistochemická analýza CD9, CD29 a epitelo-mezenchymové tranzice u triple-negativního karcinomu prsu

Ondrušček R.[1–3], Brychtová S.[1†], Bezděková M.[1] , Bouchalová K.[4], Vávrová Z.[5] , Souček K.[6], Bouchal J.[1]

[1] Department of Clinical and Molecular Pathology, Palacký University and University Hospital Olomouc, Czech Republic
[2] EUC Laboratoře CGB a.s. Ostrava, Czech Republic
[3] Department of Molecular and Clinical Pathology and Medical Genetics, University Hospital Ostrava, Czech Republic.
[4] Department of Pediatrics, Palacký University and University Hospital Olomouc, Czech Republic
[5] Department of Surgery, AGEL Hospital Ostrava-Vítkovice, Czech Republic
[6] Department of Cytokinetics, Institute of Biophysics of the Czech Academy of Sciences, Brno, Czech Republic
[†] *Deceased*

Summary

Background: Triple-negative breast carcinomas (TNBC) are a heterogeneous group of tumors with mostly aggressive behaviour and poor prognosis. In association with their aggressive behavior and chemoresistance to treatment, the concept of epithelial-mesenchymal transition (EMT) has come to the fore. CD9 and CD29 proteins are associated with EMT and may play a role in TNBC progression. Our aim was to investigate association of these markers with the lymph node metastasis, tumor grade, proliferative activity, and patient survival. ***Patients and methods:*** Our cohort consisted of 66 TNBC patients without neoadjuvant therapy, aged 26–81 years. The pathological tumor stages ranged from pT1b to pT3 and histological grades ranged from II to III, according to the Bloom-Richardson system. Immunohistochemical evaluation of CD9, CD29, E-cadherin, vimentin, androgen receptor and Ki-67 expression was performed semiquantitatively using the H-score. Expression of the proteins was statistically evaluated in relation to the clinicopathological parameters and survival of the patients. ***Results:*** We observed lower expression of CD9 in lymph node metastases compared to the primary tumor (P = 0.021). The CD29 expression in primary tumor was significantly lower in patients with lymph node metastases compared to patients without cancer dissemination (P = 0.03). Neither CD9 nor CD29 protein expression was associated with breast cancer-specific survival (BCSS). Lower expression of E-cadherin at the periphery of the primary tumor was associated with worse BCSS (P = 0.038). Neither grade nor the presence of lymph node metastases reached significant association with the BCSS. Lower expression of E-cadherin at the periphery was also associated with higher Ki67 (Rs –0.26) and vimentin (Rs –0.33). ***Conclusion:*** Decreased protein expression of CD9 and CD29 were associated with lymph node metastasis growth, however, their association with survival was not proved. Lower expression of E-cadherin at the periphery of the primary tumor was associated with high proliferation and poor breast cancer-specific survival.

Key words

triple-negative breast cancer – CD9 – CD29 – E-cadherin – epithelial-mesenchymal transition

The authors declare that they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

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doc. Mgr. Jan Bouchal, Ph.D.
Ústav klinické a molekulární patologie LF UP a FNOL
Hněvotínská 3
775 15 Olomouc
Czech Republic
e-mail: jan.bouchal@upol.cz

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Souhrn

Východiska: Triple-negativní karcinomy prsu (TNBC) jsou heterogenní skupinou nádorů s převážně agresivním chováním a špatnou prognózou. V souvislosti s jejich agresivním chováním a chemorezistencí vůči léčbě se do popředí dostal koncept epitelo-mezenchymové tranzice (EMT). Proteiny CD9 a CD29 jsou spojeny s EMT a mohou hrát roli v progresi TNBC. Naším cílem bylo prozkoumat asociaci těchto markerů s metastázami do lymfatických uzlin, gradingem tumoru, proliferační aktivitou a přežitím pacientů. ***Pacienti a metody:*** Náš soubor tvořilo 66 pacientek s TNBC bez neoadjuvantní terapie ve věku 26–81 let. Patologické stadium nádoru se pohybovalo od pT1b do pT3 a histologický stupeň od II do III podle systému Bloom-Richardson. Imunohistochemické hodnocení exprese CD9, CD29, E-cadherinu, vimentinu, androgenového receptoru a Ki-67 bylo provedeno semikvantitativně pomocí H-skóre. Exprese proteinů byla statisticky hodnocena ve vztahu ke klinicko-patologickým parametrům a přežití pacientů. ***Výsledky:*** Pozorovali jsme nižší expresi CD9 v metastázách lymfatických uzlin ve srovnání s primárním nádorem (p = 0,021). Exprese CD29 v primárním nádoru byla signifikantně nižší u pacientů s metastázami v lymfatických uzlinách ve srovnání s pacienty bez diseminace (p = 0,03). Ani exprese CD9 ani CD29 proteinu nebyla spojena s přežitím specifickým pro karcinom prsu (BCSS). Nižší exprese E-cadherinu na periferii primárního tumoru byla spojena s horším BCSS (p = 0,038). Pro grading ani přítomnost metastáz v lymfatických uzlinách nebyl nalezen signifikantní vztah s BCSS. Nižší exprese E-cadherinu na periferii byla také spojena s vyšší hladinou Ki67 (Rs –0,26) a vimentinu (Rs –0,33). ***Závěr:*** Snížená exprese proteinů CD9 a CD29 byla spojena s růstem metastáz v lymfatických uzlinách, avšak jejich souvislost s přežitím nebyla prokázána. Nižší exprese E-cadherinu na periferii primárního nádoru byla spojena s vysokou proliferací a špatným nádorově specifickým přežitím.

Klíčová slova

triple-negativní karcinom prsu – CD9 – CD29 – E-cadherin – epitelo-mezenchymová tranzice

Introduction

Mammary carcinomas stand out as the most prevalent malignancy affecting women, constituting approximately 24% of all malignancies globally [1]. Among these, triple-negative breast cancers (TNBC) form a highly diverse group of tumors, known for their aggressive nature [2]. Recurrent TNBCs result in significantly poorer long-term survival rates and an unfavorable prognosis compared to non-TNBC cases [3–6]. TNBC presents a significant clinical challenge due to its resistance to endocrine hormone therapy and other targeted treatments currently available. Ongoing research in TNBC primarily focuses on identifying novel proteins suitable for effective targeted cancer therapy [3] and discovering new prognostic markers.

Tab. 1. Clinicopathological features of triple negative breast cancer cohort (N = 66).

Parameters	N	%
Age (range 26–81 years)		
< 40	9	13.6
> 40	57	86.4
Histological subtype		
invasive cancer, no special type	53	80.3
apocrine cancer	8	12.1
adenoid cystic cancer	2	3
adenosquamous cancer	2	3
salivary like, no special type	1	1.5
Tumor size		
pT1b	10	15.2
pT1c	27	41
pT2	28	42.4
pT3	1	1.5
Tumor grade		
G1	3	4.5
G2	11	16.7
G3	52	78.8
Lymph node status		
negative	49	74.2
positive	17	25.8
Recurrence		
present	18	27.3
absent	36	54.5
not available	12	18.2
Survival	66	
dead	12	18.2
dead from cancer	10	15.2
alive	42	63.6
not available	12	18.2

One prominent exosomal marker under investigation is the tetraspanin protein CD9, which plays a crucial role in modulating cell adhesion, migration, proliferation, and vesicular transport processes, including exosomes [7,8]. CD9 plays a key role in interactions between tumor cells and the stromal microenvironment and has a major impact on tumor growth and metastasis. We have recently reviewed all immunohistochemical studies in different solid cancers; however, we concluded that CD9 is not clearly associated with either tumor suppression or promotion [9]. Additionally, CD29, also known as β1-integrin, serves as a cell surface protein receptor encoded by the ITGB1 gene, belonging to the collagen receptor family. It is commonly referred to as an epithelial-mesenchymal transition (EMT) marker [10]. CD29 regulates various biological processes, including cell proliferation, survival, and migration [11,12].

EMT, the epithelial-mesenchymal transition, is a phenomenon closely associated with malignant tumor progression and metastasis [13]. It enables a polarized epithelial cell, typically anchored to the basement membrane, to undergo biochemical changes, leading to the adoption of a mesenchymal cell phenotype. This transformation includes heightened migratory capacity, invasiveness, increased resistance to apoptosis, and significantly augmented production of extracellular matrix compo-

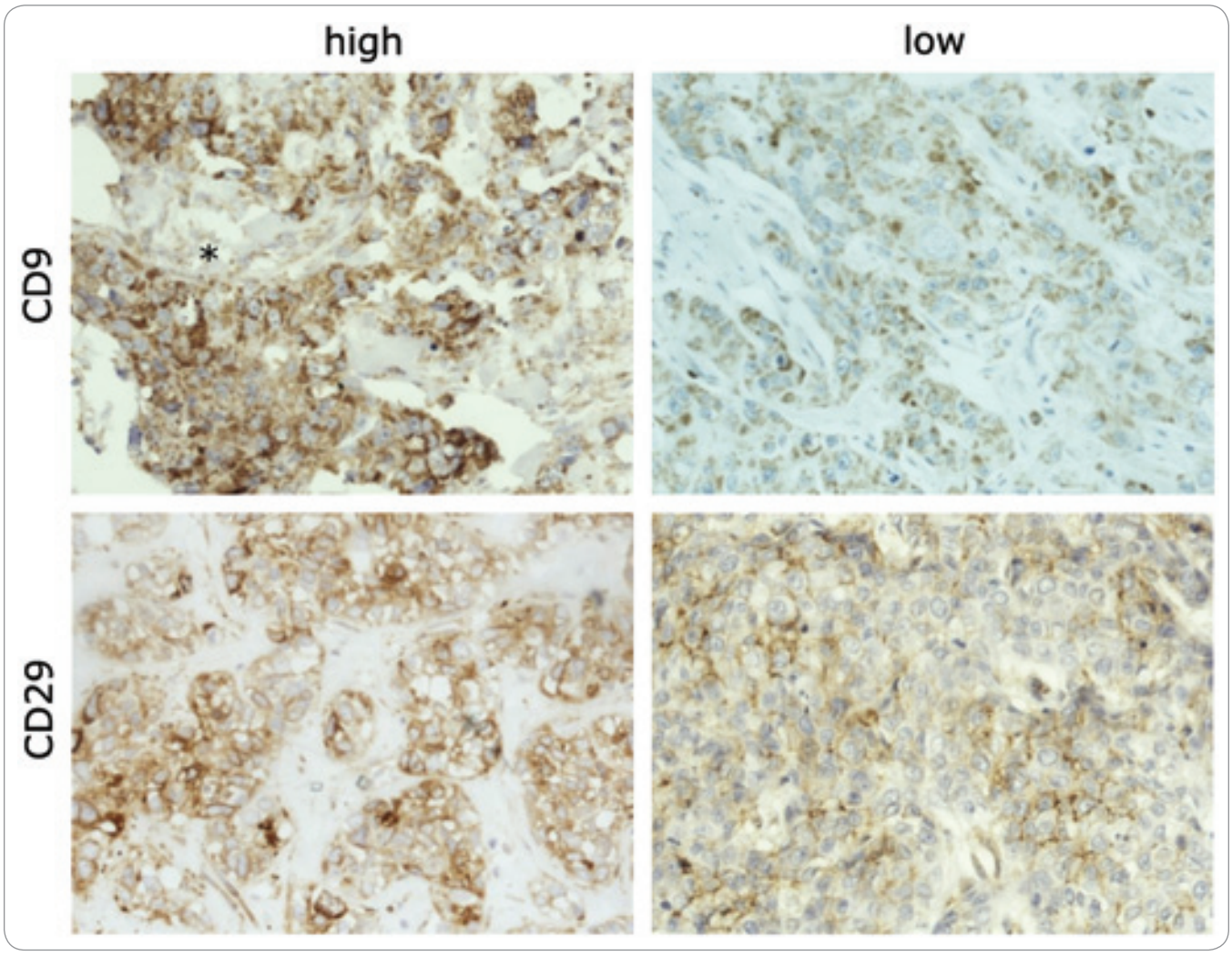


Fig. 1. An example of high expression of CD9 in the primary tumor and low level in the lymph node metastasis from the same patient. Extracellular positivity of CD9 was occasionally observed (indicated by asterisk). The lower panel shows an example of high CD29 expression in a primary tumor without dissemination and low expression of CD29 in a patient with lymph node metastasis. Magnification 100×.

nents, collectively forming the tumor microenvironment [13–15]. EMT frequently accompanies the progression of TNBC and contributes to its resistance to cancer therapy [2].

We have previously found by flow cytometry a decreased CD9 and CD29 expression in breast cancer cells that underwent EMT [16]. We now aimed to investigate CD9 and CD29 expression along with E-cadherin and vimentin (EMT markers) in a cohort of 66 TNBC patients without neoadjuvant therapy. The formalin-fixed paraffin-embedded (FFPE) samples from primary tumors were carefully selected and lymph node metastases were also included from 17 patients. Importantly, information on the clinical follow-up and survival of the patients was chased up.

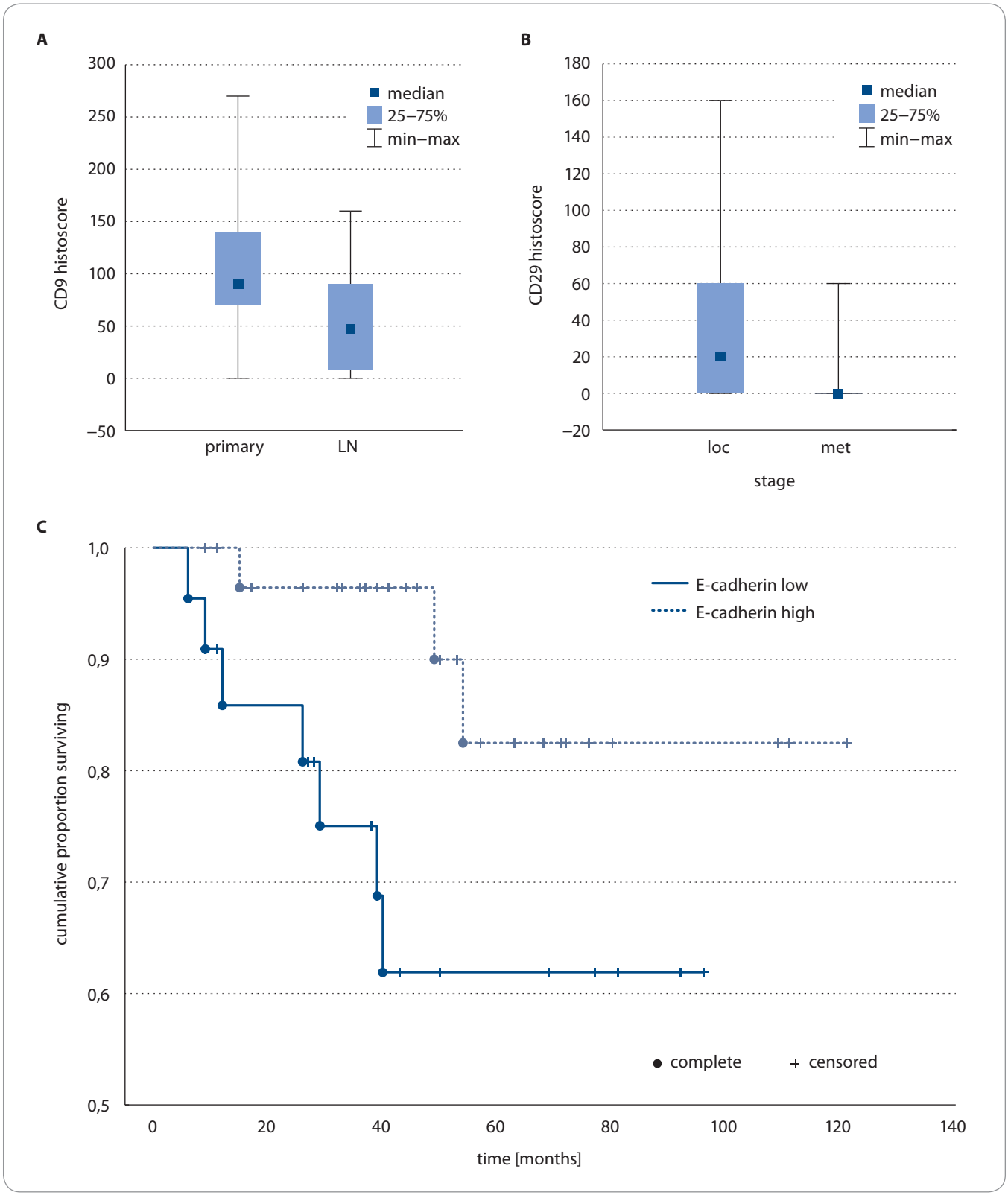
Patients and methods

Patients characteristics

Our cohort (Tab. 1) consisted of 66 patients with triple-negative breast cancer diagnosed from biopsy of surgical specimens of breast (quadrantectomy or mastectomy samples from University Hospital Ostrava, AGEL Hospital Ostrava-Vítkovice, Hospital Karviná-Ráj and EUC Klinika Kladno), which were examined during the years 2013–2022. The patients' age ranged from 26 to 81 years, with pathological tumor stages ranging from pT1b to pT3 and histological grades ranging from II to III according to the Bloom-Richardson system. Patients who received neoadjuvant therapy were excluded from the study. TNBC was defined as carcinomas showing simultaneous immunohistochemical negativity for estrogen and progesterone receptors, Her2/neu, and confirmed negativity through genetic fluorescent *in situ* hybridization (FISH). The series included 53 tumors with invasive carcinoma of the NST type, 8 apocrine carcinomas, 2 adenoid cystic carcinomas, 2 adenosquamous carcinomas, and 1 "salivary-like" carcinoma without further specification.

Immunohistochemistry

Tissue samples were fixed in 10% formalin and embedded in paraffin, then they



Graph 1. Relationship of CD9, CD29 and E-cadherin to lymph node metastasis and breast cancer specific survival (BCSS). A) The expression of CD9 was significantly higher in primary tumors compared to lymph node metastases (P = 0.021). B) The expression of CD29 was significantly higher in patients with localized disease compared to tumors with lymph node metastases (P = 0.030). C) The lower expression of E-cadherin at the periphery of the primary tumor was associated with worse BCSS (P = 0.038). The box-plots represent 25–75 percentiles, median and range of values.
LN – lymph nodes

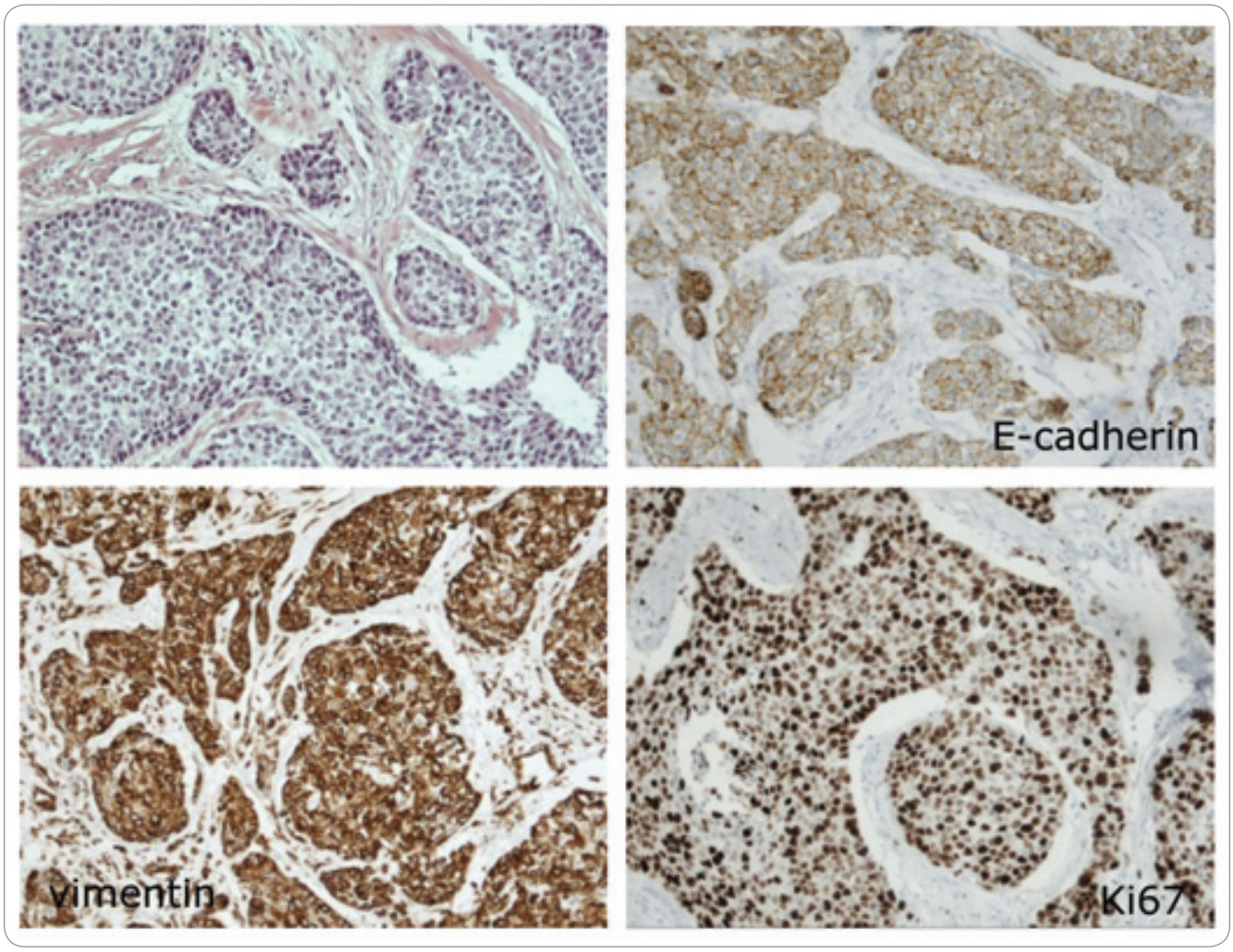


Fig. 2. An example of triple negative breast cancer. The tumor consists of solid groups of cells and displays low expression of E-cadherin at the periphery of the tumor and high expression of vimentin and Ki67. Magnification 100× (for hematoxylin-eosin staining and for immunohistochemical staining of proteins).

were cut into 2–3-µm sections. Selected proteins were investigated by indirect immunohistochemistry using specific monoclonal antibodies: rabbit anti-CD9 (clone EPR 2949, diluted 1 : 2,000; Abcam), monoclonal rabbit CD29 antibodies (clone EP1041Y, diluted 1 : 2,000; Abcam), mouse anti-E-cadherin (clone NCH-38, diluted 1 : 50, Dako), mouse anti-vimentin (clone V9, diluted 1 : 100, Dako), and anti-Ki67 (clone 30-9, Ventana).

The immunohistochemical assessment of CD9 and CD29 expression was performed semiquantitatively using the H (histo) score, which included percentage of positivity and a 4-level grading of staining intensity: 0 – no expression, 1– low intensity, 2 – moderate intensity, 3 – strong intensity. Protein expression was monitored in the tumor center and its periphery, both in tumor cells and stroma, simultaneously with its presence in lymphocytes and further in the surrounding non-tumor breast tissue.

Statistical analysis

The results were statistically evaluated using the Mann-Whitney U test, the Wilcoxon test, and the Spearman's rank correlation coefficient along with the Kaplan-Meier survival analysis with the log-rank test (STATISTICA 12, TIBCO Software).

Results

An immunohistochemical study was conducted on a cohort of 66 TNBC patients without neoadjuvant therapy (Tab. 1). Most patients with high-grade tumors received adjuvant chemotherapy with anthracycline-cyclophosphamide and taxanes. One patient was treated with carboplatin and gemcitabine, and two patients received capecitabine. Information on the clinical follow-up and survival of the patients was chased up. Breast cancer specific survival (BCSS) was defined as the time from diagnosis to death from breast cancer, while overall survival (OS) was related to death from any cause.

We found a significant decrease in CD9 expression in nodal metastases in comparison to primary carcinomas ($P = 0.021$) (Fig. 1 Graph 1A). Interestingly, we also observed an extracellular CD9 positivity (Fig. 1) which was manifested in majority of grade 3 tumors

Tab. 2. Major findings of the study.

CD9
We found a significant decrease in CD9 expression in nodal metastases in comparison to primary carcinomas.
The extracellular CD9 positivity was manifested in majority of grade 3 tumors (16 out of 17 cases).
CD29
The predominant membrane expression of CD29 in tumor cells was significantly lower in carcinomas forming lymphatic metastases compared to localized pTN0 tumors.
E-cadherin
The diminished E-cadherin expression at the periphery correlated with higher Ki67 and vimentin levels.
The reduced E-cadherin at the periphery of primary tumors was associated with poor breast cancer-specific survival.
Ki67
The strong association with high grade of tumor was observed for high Ki67 expression.

(16 out of 17 cases); however, it did not associate with nodal positivity or higher pT stages. Expression of CD9 was also found in lymphocytes, which was significantly higher in grade 3 tumors in comparison with grades 1 and 2 (P = 0.011). As expected, strong association with a high grade was observed for a high Ki67 proliferation marker (P < 0.001).

The CD29 marker demonstrated both cytoplasmic and membrane expression. The predominant membrane expression of CD29 in tumor cells was significantly lower in carcinomas forming lymphatic metastases (P = 0.030) compared to localized pTN0 tumors (Fig. 1 and Graph 1B). Neither CD29 nor CD9 expression was associated with survival. Importantly, reduced E-cadherin expression at the periphery of primary tumors correlated with poor BCSS (P = 0.038) (Graph 1C and Fig. 2). Moreover, we established associations between E-cadherin, vimentin, and proliferation marker Ki67. Diminished E-cadherin expression at the periphery correlated with higher Ki67 (Rs –0.26) and vimentin (Rs –0.33) levels (Fig. 2). The most important results are also summarized in Tab. 2.

Discussion

Our study unveiled a diminished expression of CD9 in nodal metastases, consistent with existing literature [17,18]. CD9 has been extensively studied as a prognostic marker for solid tumors. Majority of studies indicate a worse prognosis for CD9-low tumors compared to those with high expression [19–24]. However, conflicting results have also been documented [25–27]. Although we observed low expression in lymph node metastases, CD9 was not significantly associated with breast cancer specific survival.

We have also observed an extracellular CD9 positivity which may be explained by CD9 presence in membranes of exosomes, microvesicles, or apoptotic bodies [28]. Further exploration could involve alternative staining methods like TUNEL for apoptosis, or monitoring other markers of exosomes and microvesicles.

Another significant finding of our study is the association of low CD29 expression with positivity of lymph node metastases. This may agree with our previous observation of a decreased CD9 and CD29 expression in breast cancer cells that underwent EMT [16]. In this sense, loss of CD29 attenuated breast tumor growth but markedly enhanced tumor cell dissemination to the lungs [29]. These findings reveal that CD29 control a signaling network that promotes an epithelial phenotype and suppresses dissemination and indicate that targeting β1-integrins may have undesirable effects in TNBC. Still, other studies indicate worse survival of TNBC patients with high CD29 expression and targeted therapy is being tested [30,31]

The prominent marker of EMT is loss of E-cadherin which was associated with worse breast cancer specific survival in our study. Diminished E-cadherin expression at the periphery also correlated with higher Ki67 and vimentin levels. Several other studies described poor survival of TNBC patients with low E-cadherin expression [32–34] These results are also in line with our recent mass cytometry single cell analysis of fresh TNBC tissues [35] EMT score was calculated from epithelial (EpCAM + CD49f + CD9) and mesenchymal markers (vimentin + aSMA + CD44) for each cancer cell in the sample and it well associated with proliferation and lymph node colonization.

Conclusion

Decreased expression CD9 and CD29 were associated with lymph node metastasis growth, however, their association with EMT and survival was not proved. Lower expression of E-cadherin at the periphery of the primary tumor was associated with high proliferation and poor breast cancer specific survival.

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EDITED BY
Xiuwei Yang,
University of Kentucky, United States

REVIEWED BY
Yingjun Ding,
University of Oklahoma Health Sciences Center, United States

*CORRESPONDENCE
Jan Bouchal
jan.bouchal@upol.cz

†Deceased

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Prognostic value and multifaceted roles of tetraspanin CD9 in cancer

Róbert Ondruššek[1,2], Barbora Kvokačková[3,4,5], Karolína Kryštofová[6,7], Světlana Brychtová[1†], Karel Souček[3,4,5] and Jan Bouchal[1,8*]

[1]Department of Clinical and Molecular Pathology, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czechia, [2]Department of Pathology, EUC Laboratore CGB a.s., Ostrava, Czechia, [3]Department of Cytokinetics, Institute of Biophysics of the Czech Academy of Sciences, Brno, Czechia, [4]International Clinical Research Center, St. Anne's University Hospital, Brno, Czechia, [5]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czechia, [6]Proteomics Core Facility Central European Institute of Technology, Masaryk University, Brno, Czechia, [7]National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Brno, Czechia, [8]Department of Clinical and Molecular Pathology, University Hospital Olomouc, Olomouc, Czechia

CD9 is a crucial regulator of cell adhesion in the immune system and plays important physiological roles in hematopoiesis, blood coagulation or viral and bacterial infections. It is involved in the transendothelial migration of leukocytes which might also be hijacked by cancer cells during their invasion and metastasis. CD9 is found at the cell surface and the membrane of exosomes affecting cancer progression and therapy resistance. High expression of CD9 is mostly associated with good patients outcome, with a few exceptions. Discordant findings have been reported for breast, ovarian, melanoma, pancreatic and esophageal cancer, which might be related to using different antibodies or inherent cancer heterogeneity. According to *in vitro* and *in vivo* studies, tetraspanin CD9 is not clearly associated with either tumor suppression or promotion. Further mechanistic experiments will elucidate the role of CD9 in particular cancer types and specific conditions.

KEYWORDS

CD9, cancer, immunohistochemistry, prognosis, exosomes

1 Introduction

Tetraspanin CD9, also known as TSPAN29 or motility-related protein 1, is a member of the transmembrane 4 superfamily proteins, which are characterized by four transmembrane domains, two extracellular loops, and short intracellular N-and C-terminal tails (Figure 1A) (1, 2). Like other tetraspanins, CD9 can undergo palmitoylation on each of its membrane-proximal cysteines which affects its interactions with other partners (3). Tetraspanins generally form tetraspanin-enriched microdomains (TEMs) in cell membranes. Within these domains, they interact with various

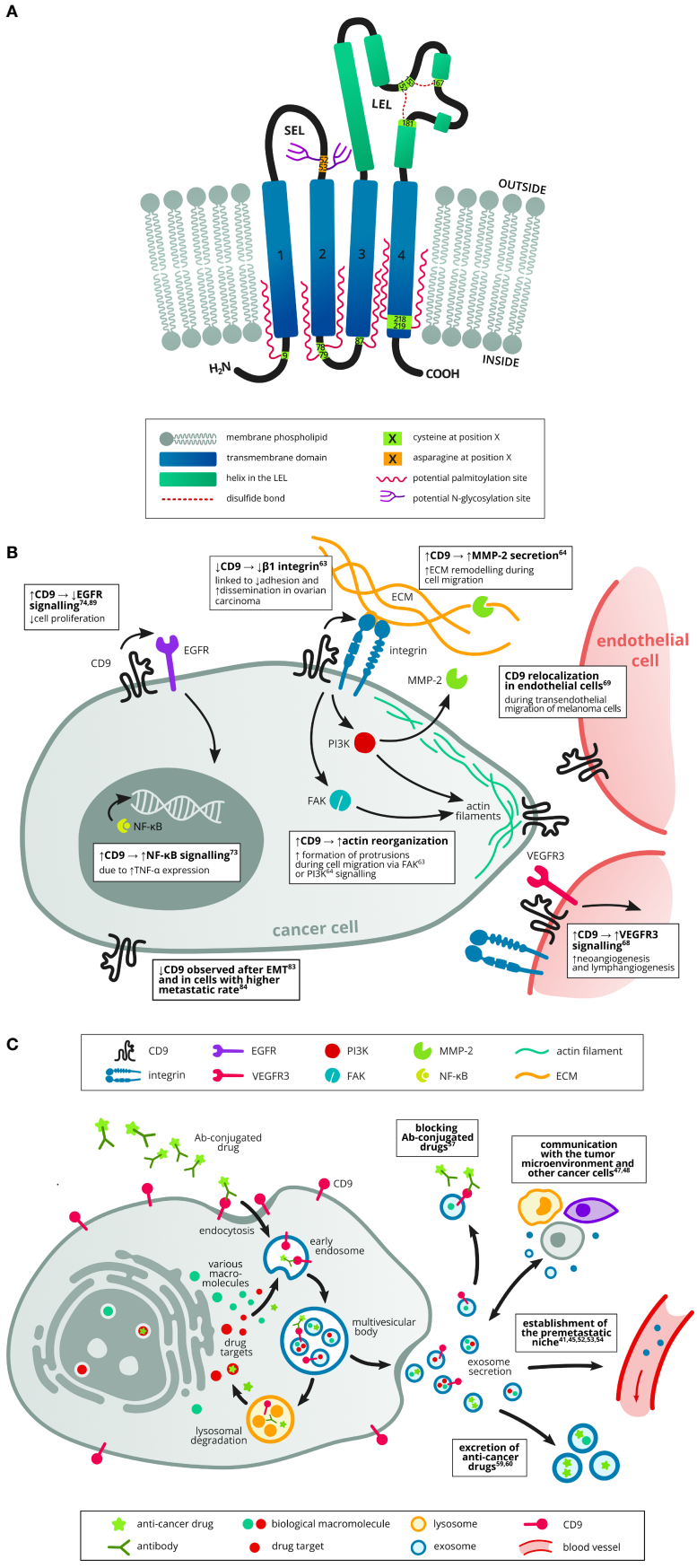

FIGURE 1 (Continued)

FIGURE 1 (Continued)
Structure of tetraspanin CD9 and its role in cancer, including exosome trafficking. **(A)** The CD9 protein consists of four transmembrane domains (1–4), short (EC1, SEL) and long (EC2, LEL) extracellular loop, short intracellular loop and short intracellular N- and C-termini. There are several possible palmitoylation sites made up of membrane-proximal cysteines and a possible N-glycosylation site in the SEL. In the LEL, there are two disulfide bridges, each containing one cysteine of the CCG motif (152–154), a typical feature of the tetraspanin family. Based on UniProt (AC: P21926, cited 1.8.2022). **(B)** CD9 was implicated both in tumor promoting and suppressing mechanisms. Several studies have described its role in cell migration and invasion, e.g. by affecting actin-polymerization and reorganisation at the cell protrusions (5, 6) or by increasing the production of the proteinase MMP-2 which cleaves ECM components during cell invasion (6). Increased CD9 expression was also linked to increased signalling in the protumorigenic NF-κB pathway (7). However, increased CD9 expression was shown to attenuate EGFR signalling and thus suppress cell proliferation (8, 9). Another study described higher metastatic rate in cells with decreased CD9 expression (10). CD9 downregulation was also observed in cells which underwent EMT (11). CD9 can also affect tumor neoangiogenesis by promoting VEGFR3 signalling in endothelial cells (12). Last but not least, transendothelial migration of tumor cells is supported by CD9 reorganisation at points of contact between endothelial and tumor cells (13). **(C)** Exosomes can transport cargo between cells in the tumor microenvironment (other tumor cells, stromal cells, immune cells) and thus enable mutual communication (14, 15). They also help establish the premetastatic niche in the target organ before colonization (16–20). Exosomes can also promote drug resistance *via* several mechanisms, e.g. by transporting drugs out of the tumor cells (21, 22) or by neutralisation of antibody-conjugated drugs (23).

transmembrane and intracellular partners, including other tetraspanins, integrins, proteases, immunoglobulins, and intracellular signaling proteins (4). Therefore, the biological effects of CD9 depend on these dynamic interactions within the context of TEMs (1, 24). Recently, a "concatenation model" for forming CD9/EWI-F assemblies has been suggested, which may explain the occurrence of these TEMs (25). Besides tetraspanins (e.g. CD63, CD81, CD151 and TSPAN4), CD9 interacts with numerous single-span transmembrane proteins, such as integrins (e.g. CD49c/ITGA3 and CD29 (1, 26), immunoglobulin superfamily proteins (e.g. EWI-F/PTGFRN and EWI-2/IGSF8) (1, 27), heparin-binding EGF-like growth factor (28), and metalloprotease ADAM17 (A Disintegrin And Metalloproteinase 17) (29). Previously, CD9 has also shown the ability to interact with other proteins such as CD19, CD46 and CD117 (30–32).

An equally important role is played by the interaction of tetraspanins with intracellular signaling molecules, although significantly fewer of them have been identified compared to transmembrane partners (4). In the context of CD9, these are mainly interactions with small GTPases of the Rho family (Rac and RhoA) that affect the actin cytoskeleton (33, 34), ERM proteins (ezrin-radixin-moesin) that mediate binding with the cytoskeleton, and PKC (35), which regulates the function of a wide range of proteins and intracellular signaling. Obviously, tetraspanin CD9 plays a complex role both in physiological conditions as well as in many diseases including cancer (Figure 1B).

2 Physiological roles of CD9

CD9 is a key regulator of cell adhesion in the immune system and plays an important role in the physiology of leukocytes and endothelial cells as well as in hematopoiesis and blood coagulation. Other physiological processes with the important role of CD9 include sperm-egg fusion (36), neurite outgrowth (37) or myotube formation (38). Recently, CD9 and tetraspanin 4 were revealed as membrane curvature sensors which play an essential role in the formation of migrasome and fertilization (39). In this sense, it was also shown that the reversed cone-like molecular shape of CD9 generates membrane curvature in the crystalline lipid layers, which explains the CD9 localization in regions with high membrane curvature and its implications in membrane remodeling (40). CD9 is also an exosomal marker and may affect the cellular exosomal transport and interactions between tumor cells and the stromal microenvironment (see chapter below).

One of the important functions of CD9 is the regulation of hematopoietic stem cell differentiation in the bone marrow and critical hematopoiesis events. CD9 is essential in megakaryocytic (41), lymphoid and myeloid differentiation (42). *In vitro* work proved that immature CD34+ bone marrow cells express high levels of CD9, while differentiated cells lose their expression (41). Also, CD9-expressing stromal cells of bone marrow affect hematopoietic cells and may be one factor that determines the degree of stem cell differentiation (43). Next, CD9 is involved in the blood clotting process, because it is a component of integral membrane proteins expressed on the cell surface and granular membranes of thrombocytes, which play an essential role in the coagulation process. It is part of an alpha 2b/beta 3 - CD9 - CD63 integrin - tetraspanin complex in activated platelets (44, 45).

The role of CD9 in different immune cells and its relevance to inflammation has recently been reviewed by Brosseau et al. (2). For example, the tetraspanin CD9 plays an important role in the immune synapse in two ways:/1/Through its association with LFA-1 on the T cell, CD9 controls the state of aggregation and adhesive capacity of this integrin./2/On the surface of the antigen-presenting cells, CD9 recruits ICAM-1 into TEMs, thus increasing its adhesive capacity (1). Its importance was also shown for the endothelial receptors such as integrin ligands ICAM-1 and VCAM-1, which facilitate leukocyte adhesion to the endothelium and their subsequent transmigration. Another important CD9 function is the inhibition of ADAM10 and ADAM17 sheddase activity, which enhances cell-cell adhesion and costimulatory capacity (1).

CD9 also affects viral (46) and bacterial infections (47, 48). Within TEMs, CD9 modulates various virus-induced processes at the membrane, including membrane fusion, viral budding and viral release. Sims et al. demonstrated that exosomes could enhance HIV-1 entry into human T and monocytic cell lines *via* exosomal tetraspanin proteins CD81 and CD9 (46). Infection by enveloped coronaviruses initiates with viral spike proteins binding to cellular receptors and is followed by proteolytic cleavage which prompts virus-cell membrane fusion. Infection, therefore, requires the proximity of receptors and proteases which ensures that virus-cell

entry occurs at the appropriate time and place. Earnest et al. showed that CD9 is crucial for condensing these receptors and proteases (DPP4 and TMPRSS2, respectively) which allows viruses to enter cells efficiently and rapidly (49, 50). Although it might be reasonable to think that SARS-CoV-2 virulence relies on CD9 activity by clustering and scaffolding receptor and protease (i.e. ACE2 and TMPRSS2, respectively) for efficient cell entry, this hypothesis has not been validated yet in current literature (50, 51).

3 CD9 in exosomes and cell-to-cell communication

There is a growing interest in cell-cell communication mediated by secreted vesicles termed exosomes (Figure 1C) (52). Exosomes are nanoscaled extracellular vesicles (EVs) (generally, their sizes range from 30 to 150 nm) released by almost all cell types. Tetraspanins are an abundant component of exosome membranes, with CD9 being one of the most frequently found along with CD63, CD81, CD82 and TSPAN8 (53, 54). Functionally it has been demonstrated that CD9 knock-down in extracellular vesicles from breast cancer cells or recipient cells reduced endocytosis (55). Importantly, tetraspanins can influence the composition of exosomes through interactions with their binding partners. For example, a decrease in CD9 expression led to a significant reduction in the metalloprotease CD10 content in exosomes of pre-B-lymphocytes (56). CD10 can serve as a positive (57) and negative (58) prognostic factor in some cancers, but its role in exosomes has not yet been described. It has also been shown that the alteration of CD9 and CD151 on prostate cells alters the proteome of their resultant EVs and that these EVs can enhance the migratory and invasive capabilities of a non-tumorigenic prostate cellular population (59). The cargo of exosomes reflects the state of tumor cells from which they are derived. They can be explored as minimally invasive biomarkers for the early detection, diagnosis and prognosis of various cancers (16, 44, 60). Currently, there are many methods for exosome isolation and detection, ranging from classical ultracentrifugation or filtration to immunoaffinity, flow cytometry and acoustics-based microfluidic techniques (61, 62).

The mechanisms of exosome biogenesis are highly regulated through several distinct pathways, including ESCRT (endosomal sorting complexes required for transport) - dependent and ESCRT-independent pathways (17). Although the exosome release is a physiological process, its increased rate and specific cargo are favorable for oncogenic progression and metastases (16). Regarding CD9, its alterations affect extracellular vesicle secretion and mitophagy in melanoma cells (63). The exosome-mediated communication is not limited to the cancer cells, it has also been shown in different cell types within the tumor microenvironment locally and distantly. Bioactive molecules, including CD9 in exosomes derived from cancer and stromal cells, provide the essential signals for the re-education of various cells and remodeling the tumor architecture (14, 15). For instance, in pancreatic ductal adenocarcinoma, CD9 mediated EV uptake from cancer-associated fibroblasts that promoted tumor development (15). In the model of colon cancer blocking EV-derived CD9 by antibody prevented the morphological transformation and migratory phenotype of cancer cells that uptake EVs (64). The CD9-positive EVs were higher in patients with prostate cancer compared to ones with benign prostate hyperplasia, and its secretion can be modulated in response to dihydrotestosterone. Importantly, siRNA knockdown of endogenous CD9 reduced cellular proliferation and expression of AR and prostate-specific antigen. However, knockdown of AR did not alter CD9 expression, implicating CD9 as an upstream regulator of AR (65). The exosomes may also determine organotropism and prepare the pre-metastatic niche in the sense of Stephen Paget´s seed and soil hypothesis (17, 66). Cancer cells from primary tumors release oncogenic biomolecules to the distant site before the cell invasion occurs, forming a pre-metastatic niche in the target organ that promotes successful metastatic outgrowth (16–20).

Therapy resistance in cancer can also occur *via* exosomes in several ways (17). Corcoran et al. reported the transfer of MDR1 by exosomes which enhanced a docetaxel efflux out of the recipient cells (67). Exosomes from drug-resistant breast cancer cells could also transmit chemoresistance do adriamycin and docetaxel by a horizontal transfer of microRNAs (68). Aung et al. have shown that tumor-derived exosomes can protect cancer cells by transporting an abundance of proteins targeted by drugs, hence neutralising the therapy effects (23). Similarly, cells of the tumor microenvironment also release exosomes that can enhance drug resistance in cancer cells. For example, fibroblast-derived exosomes have been shown to decrease the efficiency of chemotherapy and radiation in cancer by activating STAT1 and NOTCH3 signaling, which resulted in the expansion of therapy-resistant tumor-initiating cells (69). Chemotherapeutic drugs may also be excreted from cancer cells *via* exosomes (21, 22). Together, these studies described various mechanisms of exosome-mediated drug resistance either through pumping anticancer drugs out of cells or transferring molecular cargo between cells.

4 CD9 in cancer cells: Dr. Jekyll and Mr. Hyde

CD9 expression is deregulated in a number of pathologies, including cancer, but the precise mechanism underlying these changes and the associated consequences are not fully understood (4). Bioinformatic analysis of binding sites in the promoter/enhancer region of the CD9 gene identified E2F, NFkB, SP1 and STAT3 as top transcription factors often associated with both the process of carcinogenesis and disease prognosis (70). Nevertheless, the CD9 protein plays a dual role in cancer progression, exhibiting both tumor-supportive and tumor-suppressive properties that are context-dependent.

4.1 Tumor-promoting properties

The plasma membrane protrusions enable the spreading of neoplastic cells, helping them to move between and invade surrounding stromal cells. In addition to passing through intercellular gap junctions, the neoplastic cell can also use a transcellular route for intra/extravasation, which are the essential steps of the metastatic process (71). Overexpression of CD9 has been shown to enhance FAK phosphorylation and reorganisation of the cortical actin cytoskeleton in fibrosarcoma HT1080 cells plated on laminin (5). This was also associated with the induction of MMP2 and PI3K-dependent signaling (6). Aggressive triple-negative breast cancer MDA-MB-231 cells displayed significant alterations of their plasma membrane protrusions after CD9 knockdown and had reduced tumorigenic and metastatic capacity in mouse xenografts (72). The potential role of CD9 in metastasis was indicated in a different study, describing increased expression of CD9 in breast cancer bone metastasis compared to primary tumors, where CD9 antibody treatment *in vivo* moderately inhibited the progression of bone lesions (73). In this sense, CD9 expression and migration were induced by native type IV collagen through a DDR1-dependent pathway in the breast cancer model, but not in non-tumorigenic MCF10A and MCF12A cells (66). Surprisingly, along with the plasma membrane, CD9 can also localize in nuclei and its depletion led to polynucleation and multipolar mitosis (74).

CD9 has also been shown to interact with VEGFR3 signaling. After intrathoracic implantation of lung cancer cells, metastasis to lymph nodes was diminished and accompanied by decreased neoangiogenesis and lymphangiogenesis in CD9 knock-out mice (12). Knocking down CD9 in human lymphatic endothelial cells also decreased their migration, proliferation and tube formation which was associated with attenuated VEGFR3 signaling. Importantly, active redistribution of endothelial CD9 was also observed during interactions between melanoma and endothelial cells in an intravasation assay (13). Anti-CD9 monoclonal antibodies specifically inhibited the transendothelial migration of melanoma cells. Association of CD9 with transendothelial invasion has also been observed by immunohistochemistry in cervical cancer as well as in melanomas (75, 76). Similarly, Hori et al. found CD9 expression at severe vessel invasion in gastric cancer (77). CD9 upregulation was also detected in ovarian carcinomas by expression profiling and immunohistochemistry (7). The CD9 upregulation associated with enhanced expression of TNF-alpha and NFkB signaling and treatment with CD9 blocking antibody ALB6 resulted in reduced tumor growth *in-vivo* (7). CD9 can also attenuate EGF signaling pathways in gastrointestinal cancer cells by colocalizing with EGFR (8).

One of the key drivers of tumor progression are cancer stem cells (CSCs) capable to self-differentiate, self-renew and fueling tumor growth. It has been reported that CD9 identifies a subpopulation of pancreatic cancer stem cells (CSCs) able to initiate and sustain pancreatic cancer growth as demonstrated in CD9 deficient organoid and mice models (78). Mechanistically, CD9 promoted the plasma membrane localisation of the glutamine transporter ASCT2, enhancing glutamine uptake in cancer cells. CD9 has been identified as a marker of CSCs also in the glioblastoma model, where its disruption led to decreased cell proliferation, invasion, and inhibition of tumor growth (79, 80). Decreased cell migration was also reported in highly metastatic hepatocellular carcinoma cells upon CD9 silencing (81) or in breast cancer cells using CD9-binding peptide (82). The same group successfully reduced melanoma lung metastasis after peptide binding to tetraspanin CD9. The CD9-binding peptide impeded tetraspanin web formation, cancer cell invasion and significantly reduced secretion and uptake of cancer cell exosomes (83). Antibody targeting of CD9 in pancreatic cancer disrupted CD9/ADAM interactions and led to decreased proliferation, migration and colony formation (84).

Regarding small-cell lung cancer (SCLC), CD9 was expressed preferentially in SCLC tumors and metastases from three of seven relapsed patients, whereas chemonaïve primary tumors from 16 patients were CD9 negative with only one exception (85). Mechanistically, CD9 was upregulated in chemoresistant cell lines, which adhered more tightly to fibronectin *via* β1 integrin, but they were less motile than the respective chemosensitive parental lines, implying a potential role of CD9 molecule in the cell adhesion-mediated drug resistance (85).

4.2 Tumor-inhibiting properties

Regarding breast cancer, Remsik et al. observed the downregulation of CD9 in cancer cells that underwent epithelial-mesenchymal transition (EMT) both *in vitro* and *in vivo* (11). High CD9 expression is associated with epithelial phenotype and favorable prognosis regarding recurrence-free survival (11). However, further mechanistic studies will be needed to clarify the role of CD9 in EMT and breast cancer progression. In ovarian cancer, the downregulation of CD9 attenuated the expression of several integrins and rearranged junctional and cytoskeletal molecules which was associated with weaker adhesion to the extracellular matrix (10). Enhanced peritoneal dissemination was observed for subclones with low CD9 expression (10), consistent with a previous report of inverse correlation of CD9 and ovarian cancer tumor stage (86). Decreased CD9 clustering may reflect the tendency of malignant cells to have less organized cell-cell junctions where tetraspanins are typically known to be clustered (3). Low affinity anti-CD9 antibody, C9BB, which binds preferentially to CD9 homodimer was used in experiments documenting a shift to heterodimers in cancer cells. This may be associated with decreased CD9 palmitoylation or altered expression of CD9 partners (3).

Takeda et al. observed a decreased lymph node metastasis of lung cancer cells transduced with CD9 without impact on the primary tumor growth (87). Similarly, ectopic expression of CD9 in fibrosarcoma cell line HT1080 reduced their lung metastatic ability by forming a complex with podoplanin, suppressing podoplanin-induced platelet aggregation (88). In line with these studies, genetic ablation of CD9 in a model of mouse prostate adenocarcinoma did not affect primary tumor development. Still, it increased the incidence of metastases to the liver but not the lungs,

suggesting a possible tissue-specific manner of CD9 interactions in this model (89).

The antiproliferative effect of CD9 was also observed in *in vitro* model of human glioblastoma executed *via* inhibition of EGFR phosphorylation (9). In contrast, the downregulation of CD9 promoted cancer growth and metastasis through the upregulation of EGF in pancreatic cancer (90). In the context of SCLC that develops distant metastases extremely early, Funakoshi et al. observed the downregulation of tetraspanin CD9 in all cell lines. CD9 recovery suppressed cell motility of SCLC cells, suggesting that low expression of CD9 affects cell motility and may contribute to the highly invasive and metastatic phenotype of SCLC (91). Likewise CD9 overexpression in hepatocellular carcinoma inhibited proliferation *in vitro* and *in vivo* while CD9 knockdown enhanced *in vivo* growth (92).

5 Prognostic value of CD9 in solid tumors and a problem of different antibodies

Besides studies dealing with the molecular function of CD9, expression of this gene was also monitored in large cancer patient cohorts concerning tumor aggressiveness and survival. Relevant articles since 1993 are summarized in Table 1 and some are briefly commented on below. High expression of CD9 is mostly associated with good patient outcomes, with a few exceptions. These might be attributed to special cancer subtypes and different antibodies used for CD9 staining.

Discordant findings have been reported for breast, ovarian, melanoma, pancreatic and esophageal cancer. The good prognostic value of high CD9 expression was described in studies using the in-house mouse monoclonal antibody m31-15 (93, 100) and a monoclonal antibody from Dako (94). Of note, studies using the antibody clone m31-15 consistently report the good prognostic value of high CD9 in all cancer types (see Tables 1A, 1B). On the other hand, other groups reported poor outcomes for breast and ovarian cancer patients with high CD9 expression using Abcam monoclonal antibody EPR2949 or an antibody from Millipore (7, 113, 114). Kwon et al. also evaluated stromal immune cells and their CD9 expression was associated with good patient outcome (113). The same group used the EPR2949 antibody also for colorectal cancer where the high CD9 expression in tumors was associated with a good prognosis (103). Regarding stroma, high stromal CD9 evaluated with antibody clone C-4 was also associated with better survival of patients with pancreatic ductal adenocarcinoma, while positive tumor CD9 showed opposite results (115).

Another frequently used antibody is clone 72F6 from Novocastra. High levels of CD9 evaluated with this antibody were associated with poor prognosis in gastric cancer (111), while the association with good prognosis was found for mesothelioma, cervical and prostate cancer (75, 105, 116). Several other studies used an antibody from Novocastra without closer specification, complicating their findings' interpretation. One of these studies

TABLE 1 (A) Summary of immunohistochemical studies – high CD9 expression associates with good clinical outcome.

Authors	Tumor Type	Patients	Detection method	Antibody clone	Source	Therapy	Clinical outcome with high CD9
Miyake et al., 1995 (93)	breast ca	143	IHC, RT-PCR, western blotting	m31-15	in-house	S	less aggressive disease
Arihiro et al., 1998 (94)	breast ca	93	IHC, western blotting	n.s.	Dako	S	good prognosis, low risk of LN metastases
Houle et al., 2002 (86)	ovarian ca	40	IHC	m31-15	in-house, Dr. Miyake	S/AC	less aggressive disease
Sauer et al., 2003 (75)	cervical ca	44	IHC	72F6	Novocastra	S	good prognosis
Miyamoto et al., 2001 (95)	endometrial ca	71	IHC	TP82	Temecula, CA	S	good prognosis
Wang et al., 2007 (27)	prostatic ca	167	IHC	72F6	Novocastra	S	less aggressive disease
Mhawech et al., 2003 (96)	urothelial blader ca	320	IHC	n.s.	Novocastra	TURBT	good prognosis
Ai et al., 2007 (97)	urothelial blader ca	52	IHC	n.s.	Jingmei Biotech	TURBT	good prognosis
Buim et al., 2010 (98)	oral squamous ca	179	IHC, RT-PCR	n.s.	Neomarkers	S	good prognosis
Kusukawa et al., 2001 (99)	oral squamous ca	78	IHC, western blotting	clone 007	in-house	S	good prognosis

(Continued)

TABLE 1 Continued

Authors	Tumor Type	Patients	Detection method	Antibody clone	Source	Therapy	Clinical outcome with high CD9
Uchida et al., 1999 (100)	esophageal squamous ca	108	IHC	m31-15	in-house	S	good prognosis
Zou et al., 2012 (101)	ca of gallblader	108	IHC	n.s.	Dako	S	good prognosis
Khushman et al., 2019 (102)	pancreatic ca	29	IHC	n.s.	Abcam	S	less aggressive disease
Kim et al., 2016 (103)	colorectal ca	305	IHC	EPR2949	Abcam	S/AC	good prognosis in left-side tumors only
Hashida et al. 2003 (104)	colorectal ca	146	IHC, RT-PCR	m31-15	in-house	S	good prognosis
Amatya et al., 2013 (105)	mesothelioma	112	IHC	72F6	Novus Biologicals	S/AC/RT	good prognosis
Si et Hersey 1993 (106)	melanoma	55	IHC	FMC56	in-house, Dr. Zola	S	less aggressive disease
Woegerbauer et al., 2010 (107)	Merkell cell ca	25	IHC, RT-PCR, western blotting	RDI-MCD9	Fitzgerald	S/AC/RT	good prognosis
Kohmo et al., 2010 (85)	small cell lung ca	24	IHC, RT-PCR, western blotting, flow cytometry	72F6	Novocastra	AC	good prognosis
Adachi et al., 1998 (108)	non small lung ca	172	IHC, RT-PCR	m31-15	in house	S	good prognosis
Higashiyama et al., 1995 (109)	adenoca of lung	132	IHC, RT-PCR	m31-15	in house	S	good prognosis

AC, adjuvant chemotherapy; ca, carcinoma; IHC, immunohistochemitry; LN, lymph node; NAC, neoadjuvant chemotherapy; n.s. not specified; RT, radiotherapy; S, surgery; TURBT; transuretral resection of bladder tumor.

TABLE 1 (B) Summary of immunohistochemical studies – high CD9 expression associates with poor clinical outcome.

Authors	Tumor Type	Patients	Detection method	Antibody clone	Source	Therapy	Clinical outcome with high CD9
Kim et al., 2019 (110)	papillary thyroid ca	553	IHC	n.s.	Novocastra	S	associated with LN metastases
Hori et al., 2004 (77)	gastric ca	78	IHC, nothern and western blotting	72F6 and 007	Novocastra and Dr. Mekada, resp.	S/AC	associated with advanced disease
Soyuer et al., 2010 (111)	gastric ca	49	IHC	72F6	Novocastra	S/AC	poor prognosis
Miki et al., 2018 (14)	gastric ca	619	IHC	n.s.	Life Technologies	S	poor prognosis, in particular of the scirrhous type
Huan et al., 2015 (112)	esophageal squamous ca	104	IHC, western blotting	n.s.	Santa Cruz	S	poor prognosis, associated with advanced disease
Kwon et al., 2017 (113)	breast ca	1349	IHC	EPR2949	Abcam	S/AC	poor prognosis in luminal A subtype
Baek et al., 2019 (114)	lobular breast ca	113	IHC	EPR2949	Abcam	S/AC/RT	poor prognosis
Hwang et al.2012 (7)	ovarian ca	30	IHC, microarray, RT-PCR, western blotting	n.s.	Millipore	S	associated with advanced disease
Lucarini et al., 2022 (76)	cutaneous melanoma	120	IHC	72F6	Novocastra	S	poor prognosis, associated with LN metastases
Han et al., 2022 (115)	pancreatic ca, NAC	179	IHC	C-4	Santa Cruz	NAC/S	poor prognosis

AC, adjuvant chemotherapy; ca, carcinoma; IHC, immunohistochemitry; LN, lymph node; NAC, neoadjuvant chemotherapy; n.s. not specified; RT, radiotherapy; S, surgery; TURBT; transuretral resection of bladder tumor.

investigated breast cancer samples and found no prognostic value of CD9 (117).

Overall, the interpretation of immunohistochemistry studies is problematic due to the use of different antibodies that are not thoroughly validated and essential details are missing. For example, epitopes for the most frequently used monoclonal antibodies m31-15, 72F6 and EPR2949 are unavailable. Two recent meta-analyses concluded that low CD9 expression is significantly associated with poor prognosis of cancer patients (118, 119) however, these results are oversimplified and problematic with respect to the abovementioned issues.

As described in the previous chapter, CD9 is not clearly associated with either tumor suppression or promotion. Additional mechanistic studies have recently been thoroughly reviewed by Lorico et al. They also summarized CD9 targeting with therapeutic antibodies and drew attention to the potential side effects of this strategy. This approach might further be complicated by the presence of CD9 in exosomes which may neutralize the therapeutic antibodies or enhance the pro-metastatic effects of exosomes by their enhanced endocytosis (53).

6 Conclusion

A growing body of evidence points to the important role of CD9 in various physiological processes and cancer. As highlighted in the review, CD9 is not unequivocally associated with either tumor suppression or promotion, and the antibodies used to detect CD9 might be problematic. Despite conflicting results in different types of cancer, the clinical relevance of CD9 has been highlighted by several immunohistochemical and, more importantly, mechanistic studies. Therapeutic targeting of CD9 is emerging, however, this approach may be complicated by the presence of CD9 in exosomes, which may neutralize therapeutic antibodies or enhance the pro-metastatic effects of exosomes by their increased endocytosis. In conclusion, fully validated antibodies and well-designed functional studies may help to elucidate further the role of CD9 in cancer progression and patient clinical outcome.

Author contributions

JB, SB, KS – conceptualization. RO – writing, preparation of original draft. BK, KK, KS – writing, reviewing and editing. KK - graphical figures preparation. JB - supervision, writing, reviewing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Single-cell protein profiling defines cell populations associated with triple-negative breast cancer aggressiveness

Barbora Kvokačková[1,2,3], Radek Fedr[1,2], Daniela Kužílková[4,5], Jan Stuchlý[4,5], Adéla Vávrová[4,6], Jiří Navrátil[7], Pavel Fabian[8], Róbert Ondruššek[9,10], Petra Ovesná[11], Ján Remšík[12], Jan Bouchal[9], Tomáš Kalina[4,5] and Karel Souček[1,2,3]

1 Department of Cytokinetics, Institute of Biophysics of the Czech Academy of Sciences, Brno, Czech Republic
2 International Clinical Research Center, St. Anne's University Hospital, Brno, Czech Republic
3 Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic
4 Childhood Leukaemia Investigation Prague, Czech Republic
5 Department of Pediatric Haematology and Oncology, 2nd Faculty of Medicine, Charles University Prague and University Hospital Motol, Czech Republic
6 Faculty of Science, Charles University Prague, Czech Republic
7 Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Brno, Czech Republic
8 Department of Oncological Pathology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
9 Department of Clinical and Molecular Pathology, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University and University Hospital, Olomouc, Czech Republic
10 Department of Pathology, EUC Laboratoře CGB a.s., Ostrava, Czech Republic
11 Institute of Biostatistics and Analyses, Faculty of Medicine, Masaryk University, Brno, Czech Republic
12 Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York City, NY, USA

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Correspondence
K. Souček, Institute of Biophysics, Czech Academy of Sciences, Královopolská 135, 612 00 Brno, Czech Republic
Tel: +420 541 517 166
E-mail: ksoucek@ibp.cz

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Triple-negative breast cancer (TNBC) is an aggressive and complex subtype of breast cancer that lacks targeted therapy. TNBC manifests characteristic, extensive intratumoral heterogeneity that promotes disease progression and influences drug response. Single-cell techniques in combination with next-generation computation provide an unprecedented opportunity to identify molecular events with therapeutic potential. Here, we describe the generation of a comprehensive mass cytometry panel for multiparametric detection of 23 phenotypic markers and 13 signaling molecules. This single-cell proteomic approach allowed us to explore the landscape of TNBC heterogeneity, with particular emphasis on the tumor microenvironment. We prospectively profiled freshly resected tumors from 26 TNBC patients. These tumors contained phenotypically distinct subpopulations of cancer and stromal cells that were associated with the patient's clinical status at the time of surgery. We further classified the epithelial-mesenchymal plasticity of tumor cells, and molecularly defined phenotypically diverse populations of tumor-associated stroma. Furthermore, in a retrospective tissue-microarray TNBC cohort, we showed that the level of CD97 at the time of surgery has prognostic potential.

Abbreviations
CAF, cancer-associated fibroblasts; CSC, cancer-stem cells; E/M, epithelial-mesenchymal; EMT, epithelial-mesenchymal transition; IHC, immunohistochemistry; LNR, lymph node ratio; MET, mesenchymal-epithelial transition; PBMCs, peripheral blood mononuclear cells; TMA, tissue microarray; TME, tumor microenvironment; TNBC, triple-negative breast cancer; t-SNE, t-distributed stochastic neighbor embedding.

1. Introduction

Triple-negative breast cancer (TNBC) is a profoundly heterogeneous subtype of breast cancer with characteristic aggressive behavior and poor outcome [1,2]. Because TNBC lacks the expression of estrogen (ER), progesterone (PR), and human epidermal growth factor 2 (HER2) receptors, cytotoxic chemotherapy remains the treatment of choice for early-stage and advanced TNBC. However, multiple clinical trials with targeted approaches and immunotherapies are ongoing [3].

Intra- and intertumoral heterogeneity are major obstacles in the effective clinical management of patients. Both were in part molecularly dissected by a number of studies that identified multiple TNBC subtypes, with each exhibiting unique biological features [4]. The intratumoral heterogeneity is driven by genetic, epigenetic, and phenotypic inputs within the cancer cells and extrinsic factors from the tumor microenvironment (TME). Such heterogeneity can then influence tumor progression and therapeutic response [5]. Phenotypic plasticity of TNBC is often attributed to epithelial-mesenchymal transition (EMT), a mechanism that generates a subpopulation of highly motile cells, often with increased ability to seed new tumors and capacity to self-renew and differentiate [6,7]. EMT and its reverse program mesenchymal-epithelial transition (MET) are key advantageous developmental programs hijacked by tumor cells to support their dissemination from the primary site. While EMT and acquisition of mesenchymal phenotype (M) is critical for cancer cell spread, subsequent reversion back to the epithelial state (E) is a prerequisite for successful metastatic outgrowth at distant sites [5,8,9].

Selected tumor cell populations can dynamically switch between EMT and MET and exist in a spectrum of hybrid E/M states, partially bearing features of both phenotypes. Cells in these hybrid states are presumed to have the highest tumorigenic and metastatic potential [10,11]. Both EMT/MET programs are influenced by a number of factors and signaling molecules, including the stereotypical EMT inducer TGF-β and SMAD signaling pathways, NF-κB, JAK–STAT proteins, PI3K/AKT/mTOR or Wnt [5,12]. These stimuli are usually governed by TME, comprised of different populations of infiltrating immune cells, stromal cells such as fibroblasts, pericytes, endothelium, and other cell types together with extracellular matrix [13]. Various elements of TME play both pro- and anti-tumor roles, and TME represents a promising therapeutic target [13,14]. An ample effort is currently being made toward the detailed characterization of tumor-TME interactions at single-cell and spatial levels. Several recent single-cell transcriptomic reports further confirmed that TNBC tumors consist of distinct subpopulations of tumor cells [15,16], cancer-associated fibroblasts [17], and immune cells [18] with clinically relevant transcriptomic signatures. The observed gene expression changes are not always reflected at the level of protein expression. Mass cytometry overcomes this limitation by employing metal-conjugated antibodies, enabling the quantification of dozens of proteins and phospho-epitopes in individual cells simultaneously [19].

In this study, we applied such mass cytometric profiling to a cohort of prospective TNBC patients. We comprehensively mapped phenotypic TNBC diversity and signaling status at the protein level. Our data revealed the presence of distinct cancer and stromal phenotypes within TNBC tumors, and their association with patient clinical status. Validation of selected markers in expanded retrospective cohorts with routine histology techniques further corroborated their stratifying potential.

2. Materials and methods

2.1. Cell lines

Breast cancer cell line MDA-MB-231 (RRID:CVCL_0062) was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and used as an internal control. Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA, TFS) supplemented with 10% fetal bovine serum (TFS) and 100 $U \cdot mL^{-1}$ penicillin/streptomycin (Sigma-Aldrich, Merck-Millipore, Darmstadt, Germany). Cells were maintained at 37 °C and 5% CO_2, routinely tested for mycoplasma contamination with PCR and authenticated using AmpFLSTR Identifiler Plus PCR Amplification Kit (TFS) to verify their origin. Peripheral blood mononuclear cells (PBMCs) were obtained from a healthy donor with written informed consent in accordance with the Declaration of Helsinki. PBMCs were isolated as a buffy coat layer on Ficol-Paque gradient. Isolated PBMCs and the MDA-MB-231 cell line were incubated with CD24 and ROR1 antibodies for 30 min at room temperature (Table 1). After washing once with Maxpar Cell Staining Buffer (CSB) (Fluidigm, South San Francisco, CA, USA), cells were stained with 1 μM cisplatin for subsequent dead cell exclusion

Table 1. Overview of antibodies and reagents used in study.

Mass cytometry

Marker	Clone	Metal	Source	Cat. no; home-made LOT identifier
CD45	HI30	Y89	Fluidigm	3089003B
CD28	CD28.2	142Nd	Bxcell	BE0291; 180116
CD49f	GoH3	143Nd	Biolegend	313602; 180906
CD69	FN50	144Nd	Fluidigm	3144018B
CD4	RPA-T4	145Nd	Fluidigm	3145001B
CD8a	RPA-T8	146Nd	Fluidigm	3146001B
ITGB5	AST-3 T	147Sm	Biolegend	345202; 180919
CD111	R1.302	148Nd	Biolegend	340402;180906
CD38	HIT2	149Sm	Exbio	11-366-C100, 190425
CD112	TX31	150Nd	Biolegend	337402; 180919
EpCAM	9C4	152Sm	Biolegend	324229; 180919
CD29	TS2/16	156Gd	Fluidigm	3156007B
ROR-1	2A2	159Tb	Miltenyi Biotec	130-98-243; 191209
CD14	M5E2	160Gd	Fluidigm	3160001B
CD49c	ASC-1	162Dy	Biolegend	343802; 180906
CD24	ML5	165Ho	Biolegend	311127; 181129
CD44	BJ18	166Er	Fluidigm	3166001B
CD90	5E10	167Er	Biolegend	328129; 180919
CD19	HIB19	169Tm	Fluidigm	3169011B
CD3	UCHT1	170Er	Fluidigm	3170001B
CD97	VIM3b	171Yb	Fluidigm	3171015B
CD9	SN4_C3-3A2	172Yb	Fluidigm	3172010B
HLA-DR	L243	174Yb	Fluidigm	3174001B
CD31	MEM-05	175Lu	Exbio	11-273-C100; 190516
CD56 (NCAM)	NCAM16.2	176Yb	Fluidigm	3176008B
αSMA	1A4	141Pr	Fluidigm	3141017D
pSmad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad9 (Ser465/467)	D5B10	151Eu	Cell Signaling	#12428; 191209
Stat1 (Y701)	58D6	153Eu	Fluidigm	3153003A
Vimentin	D21H3	154Sm	Fluidigm	3154014A
pSmad2 (Ser465/Ser467)	E8F3R	155Gd	Cell Signaling	#18338; 181129
pStat3 (Y70)	4/P-STAT3	158Gd	Fluidigm	3158005A
pNF-kBp65 (S536)	93H1	161Dy	Cell Signaling	#3033;170313
pAkt (S473)	D9E	163Dy	Cell Signaling	#4060; 170313
Active β-catenin (Ser45)	D2U8Y	164Dy	Cell Signaling	#19807; 181129
Ki-67	B56	168Er	Fluidigm	3168007B
PanCK	C-11	173Yb	Exbio	11-108-C100; 180919
CD298	REA217	N/A	Miltenyi	130-122-331; N/A
HLA-I	W6/32	N/A	Bxcell	BE0079; N/A
Chemicals				
Platinum (^{195}Pt, ^{196}Pt)			Fluidigm	201195, 201196
Cadmium (^{106}Cd, ^{110}Cd, ^{111}Cd, ^{112}Cd, ^{113}Cd, ^{114}Cd and ^{116}Cd)			Fluidigm	N/A
CisPt		198Pt	Fluidigm	201198
Iridium (^{191}Ir, ^{193}Ir)			Fluidigm	201192A

Immunohistochemistry

Marker	Clone	Host	Source	Cat. no
EpCAM	Ber-Ep-4	Mouse	DCS	EI751C002
CD49f	Polyclonal	Rabbit	Invitrogen	PA5-82466
Vimentin	SP20	Rabbit	DCS	VI686C002

Table 1. (Continued).

Immunohistochemistry				
Marker	Clone	Host	Source	Cat. no
CD90	D3V8A	Rabbit	Cell Signaling	13801
αSMA	1A4	Mouse	Dako	M0851
HLA-DR	TAL. 1B5	Mouse	Dako	M0746
NF-kB p65	F-6	Mouse	Santa Cruz Biotechnology	sc-8008
CD49c (ITGA3)	polyclonal	Rabbit	Sigma-Aldrich	HPA008572
CD97	EPR4427	Rabbit	Abcam	ab108368
AR	AR 441	Mouse	Dako	M3562
CK5/6	D5/16 B4	Mouse	Roche	790-4554
E-cadherin	NCH-38	Mouse	Dako	M3612
CD8	SP16	Rabbit	Thermo Fisher Scientific	RM-9116-S1

(Fluidigm) and quenched with CSB buffer. Cells were then fixed with 1.6% paraformaldehyde (TFS) for 15 min at room temperature and stored at −80 °C in 10% glycerol in fetal bovine serum.

2.2. Patient samples

Fresh breast cancer tissues from patients undergoing surgical breast cancer removal, in excess of that required for diagnostic and therapeutic procedures, were obtained from Masaryk Memorial Cancer Institute (MMCI) between 2018 and 2021 (Table S1). Tissues were evaluated by a certified breast cancer pathologist. The study was approved by the Ethical Committee of the MMCI (Ref. No. 2017/1894/MOU). The experiments were conducted with the understanding and written consent of each patient.

2.3. Breast cancer tissue processing

Tissue samples were minced to 1–2 mm pieces and enzymatically digested in DMEM/F12 (Gibco, TFS) containing 2% bovine serum albumin (BSA; Serva, Heidelberg, Germany), 5 $mg{\cdot}mL^{-1}$ recombinant human insulin (Sigma-Aldrich), 0.5 $mg{\cdot}mL^{-1}$ hydrocortisone (Sigma-Aldrich), 50 $mg{\cdot}mL^{-1}$ gentamicin (Serva), 2 $mg{\cdot}mL^{-1}$ collagenase type I (cat. no. LS 004194; Worthington, Lakewood, NJ, USA), 0.6 $U{\cdot}mL^{-1}$ dispase II (cat. no. 04942078001; Roche, Basel, Switzerland) and 10 mM Y-27632 dihydrochloride (ROCK inhibitor, Santa Cruz Biotechnology, Dallas, TX, USA), for 14 h at 37 °C using 10 rpm agitation. Samples were then treated with 15 $mg{\cdot}mL^{-1}$ DNase I (cat. no. 04942078001; Roche) for 5 min at 37 °C, washed with PBS, and filtered through a 70 μm strainer. Red blood cells were lysed with ACK buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, and 100 μM EDTA solution in sterile MQ water) at 37 °C for 5 min, washed with PBS and incubated with CD24 and ROR1 antibodies 30 min at room temperature. After washing once with CSB buffer, cells were stained with 1 μM cisplatin for subsequent dead cell exclusion (Fluidigm) and quenched with CSB buffer. Cells were then fixed with 1.6% paraformaldehyde (TFS) for 15 min at room temperature and stored at −80 °C in 10% glycerol in fetal bovine serum.

2.4. Mass cytometry barcoding

To achieve parallel sample analysis and thus minimize the batch effect, we used metal tagged antibody-based barcoding approach. 0.1–1 × 10^6 cells from each dissociated tumor sample, as well as MDA-MB-231 cells and PBMCs (internal controls) were barcoded using a barcoding scheme consisting of combinations of HLA-I and CD298 antibodies conjugated to platinum (^{195}Pt and ^{196}Pt) and cadmium (^{106}Cd, ^{110}Cd, ^{111}Cd, ^{112}Cd, ^{113}Cd, ^{114}Cd, and ^{116}Cd) isotopes (see Table 1). Antibodies were purchased from Fluidigm or conjugated in-house using Maxpar antibody conjugation kits (Fluidigm). Thawed individual tumor samples, cell line, and PBMCs were incubated with antibodies for 30 min at room temperature, washed with CSB buffer, and pooled together for subsequent antibody staining.

2.5. Antibodies and antibody labeling

All antibodies, clones, metal tags, and providers are listed in Table 1. Metal-labeled antibodies were prepared using the Maxpar antibody conjugation kits (Fluidigm), according to the manufacturer's instructions or purchased pre-conjugated. Each antibody was titrated and validated into the working panel to achieve an optimal signal-to-noise ratio.

2.6. Mass cytometry antibody staining and detection

After barcoding, the pooled samples were stained with a mastermix of surface antibodies for 30 min at room temperature; except for CD24 and ROR1 that were stained before fixation and freezing at −80 °C. Samples were then washed twice with CSB buffer. Next, the cells were permeabilized with 80% methanol for 30 min on ice and washed with CSB buffer. For intracellular staining, the cells were incubated with a mastermix of intracellular antibodies for 30 min at RT and then washed twice with CSB buffer. Lastly, samples were resuspended in 1 mL of Cell-ID Intercalator-Iridium in MaxPar Fix & Perm Buffer (Fluidigm). Cells were then washed twice with CSB buffer, once MiliQ H_2O, resuspended in 20% EQ Four Element Calibration Beads (Fluidigm) in MiliQ H_2O, and filtered through a 40 μm filter cap. Sample analysis was acquired on Helios™, a CyTOF® system (Fluidigm) with all opened channels and data collected as .fcs files.

2.7. Data preprocessing and analysis

Data were normalized in FLUIDIGM software based on EQ beads and compensated for channel crosstalk, as previously described [20]. These compensated .fcs files were processed with FLOWJO software (v10.8.0, BD). Cells were gated for singlets, de-barcoded, and cisplatin-positive dead cells were excluded from the analysis. The following major cell types of interest were identified by manual gating using selected marker expression pattern: $PanCK^+/CD45^-$ cancer cells, $CD45^+PanCK^-$ immune cells, $CD90^+/PanCK^-/CD45^-$ stromal cells. Representative gating strategy is shown in Fig. S1A. The total amount of collected cells from measured samples ranged from 850 to 174 000. The resulting, clean dataset contains in total 73 532 cancer cells, 560 218 immune cells, and 156 928 stromal cells.

For the purposes of unsupervised data analysis, we utilized recently published Risk Assessment Population IDentification (RAPID) algorithm [21], enabling identification of phenotypically distinct populations and determining whether they stratify patient survival. Twenty-six manually pre-gated .fcs files were exported, and loaded into RSTUDIO (4.1.1 version) [22]. Here, each file was downsampled to the same cell number and combined into new data frame. Because some samples contained lower number of cells they were excluded for further unsupervised analysis of epithelial cells (21 samples analyzed in total) and stromal fraction (24 samples analyzed in total). This preprocessed mass cytometric data frame, together with .csv file containing annotated clinical parameters of tumor specimens, was subjected to a modified RAPID algorithm. Dimensionality reduction was calculated based on 36 markers using the R T-SNE package [23]. The following parameters were used: iteration = 10 000, perplexity 200, theta 0.9, and eta 200. The optimal number of clusters was computed based on the t-SNE map using the modified script, followed by FlowSOM clustering [24].

Because our dataset lacked information about the patient outcome, on which the RAPID algorithm is based, we introduced a Ki-67 + LNR index that consisted of clinical assessment of cancer spread to lymph nodes and Ki-67 positivity, as determined by diagnostic histology analysis for each patient. This clinically relevant Ki-67 + LNR index was then implemented for modified RAPID analysis instead of patient survival.

To calculate Ki-67 + LNR index for each patient we used two variables, LNR ("lymph node ratio" = number of positive lymph nodes over number of excised lymph nodes) and Ki-67 that was normalized to maximum value from all tumors. For each analyzed cell in the dataset, we stored also information from what tumor/patient the cell originated and the cluster number in which the cell was identified by FlowSOM. We took normalized Ki-67 and LNR values of single tumors and assigned them to each analyzed cell in dataset as new variables. Then we selected cells of separate FlowSOM clusters and calculated the means of normalized Ki-67 and LNR variables. Finally, a sum of these two means results in Ki-67 + LNR index that acquires values from 0 to 2.

Mesenchymal-epithelial (MET) score was calculated for each cancer cell. Mean intensity (MI) of selected proteins measured by mass cytometry was normalized to their corresponding maximal values obtained in the dataset. The normalized values of the final range from 0 to 1 were then used to calculate the MET score. The mass cytometry MET score was then calculated as a cumulative sum of normalized MIs from epithelial markers [EpCAM + CD49f + CD9] minus the cumulative sum of MIs from mesenchymal markers [Vimentin + αSMA + CD44] for each cancer cell in the sample. The final MET score for each patient is calculated as the mean MET score from all cancer cells in the sample and acquires values from −3 (fully mesenchymal-like phenotype) to +3 (fully epithelial-like phenotype). MET score calculation was done in R.

Correlation analysis was performed in GRAPHPAD PRISM (version 9.0.2 GraphPad Software, San Diego, CA, USA) using non-parametric Spearman's

correlation. Heatmaps were generated in the CLUSTVIS tool [25].

2.8. Tissue microarray (TMA) construction and immunohistochemistry

The first set of archival tissue samples from TNBC patients was constructed at the MMCI, and contained patient tissue collected between 2005 and 2009. The second set was also constructed at the MMCI, collected between 2012 and 2021. TNBC status was determined according to ASCO standards – threshold for the hormone receptor for IHC staining of tissue sample was 1% [26]. TMA construction and immunostaining of archival formalin-fixed, paraffin-embedded tumor samples with appropriate antibodies was done according to standard techniques established at MMCI (Table 1). Protein expression was assessed semi-quantitatively by an expert breast cancer pathologist, using the histoscore (H-score) method. In H-score, the percentage of positive cells (0–100%) is multiplied by staining intensity (0–3), resulting in a final histoscore that ranges between 0 and 300. Immunohistochemistry MET score was calculated as a cumulative H-score of [EpCAM + CD49f] proteins minus a cumulative H-score of [Vimentin + αSMA] proteins. For Burstein classification of tumor tissues we used five surrogate markers – AR, CK5/6, CD8, E-cadherin, Vimentin – in co-junction with quantification of tumor-infiltrating lymphocytes according to the relevant studies [27–29]. We then classified these samples according to Burstein's classification as luminal-androgen receptor (LAR), mesenchymal (MES), basal-like immunosuppressed (BLIS), and basal-like immunoactivated (BLIA) subtypes. TNBC subtypes for this cohort were added to Table S1 and used for further analysis.

2.9. Statistical analysis

Statistical analyses and visualizations were performed in R (4.1.1 version, R Core Team, 2021) and GRAPHPAD PRISM (version 9.0.2, GraphPad Software). Kaplan-Maier analysis was performed by the survminer R package. Illustrations were created with BioRender.

3. Results

3.1. Broad view of TNBC cellular landscape through large-scale single-cell proteomics

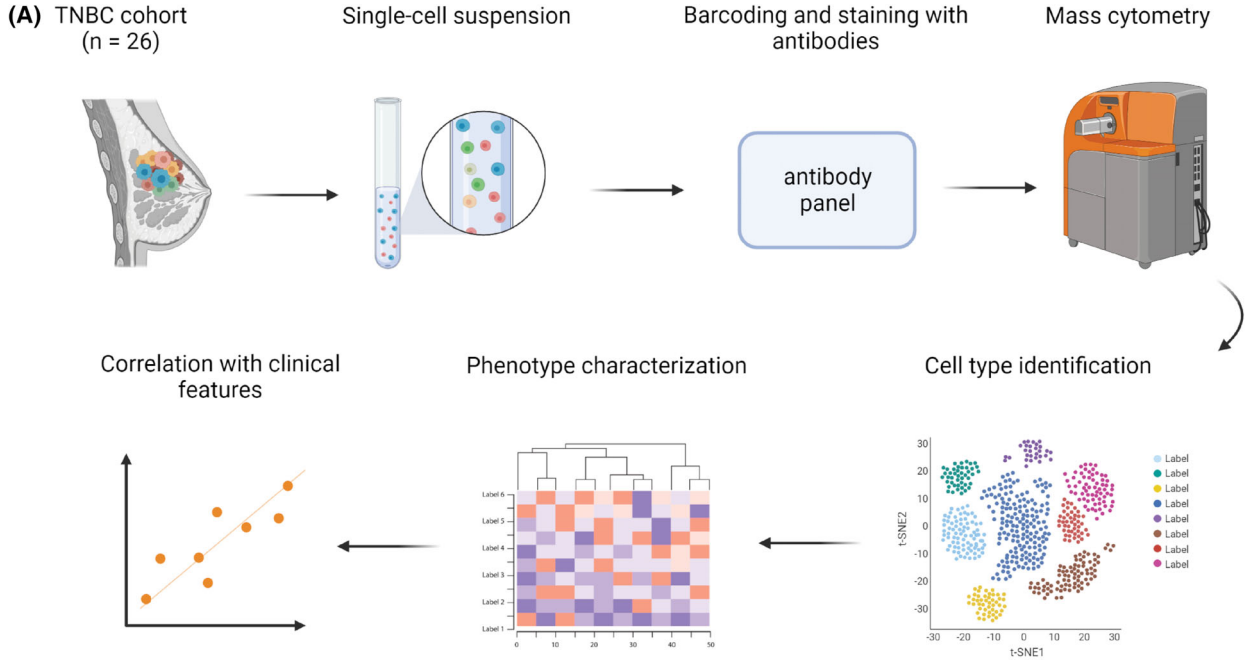
While single-cell transcriptomics vastly expanded our understanding of tumor ecosystems, this approach does not yet allow plausible profiling of cell landscapes in larger patient cohorts or greater cell numbers. To challenge this limit, we designed a prospective, single-cell-based, large-scale mass cytometric analysis of 26 treatment-naïve triple-negative breast cancer cases. Twenty-four tumors were classified as high grade 3, and two were intermediate grade 2 tumors. Six patients presented with involved lymph nodes that have been resected, and none of them had signs of metastatic disease at the time of surgery (Table S1). Freshly resected tumor samples were immediately dissociated into single cell suspensions and mass-tag barcoded, along with a cancer cell line and PBMCs that served as internal controls. Samples were then sequentially stained with a panel of surface and intracellular antibodies (Fig. 1A).

The antibody panel was designed to identify tumor cell subpopulations that are known to contribute to breast cancer progression (e.g. CD24, CD44 as markers of CSCs), surface molecules that associate with EMT/MET plasticity (e.g. EpCAM and Vimentin) and a set of novel surface antigens reflecting breast cancer cell plasticity (e.g. CD29, CD97, CD49c, ITGB5), published previously [30]. We also included markers that would allow for subtyping of stromal cells (e.g. CD90, Vimentin, and αSMA) and a spectrum of immune cell types (e.g. CD45, CD3, CD14, CD19). Additionally, to inspect the activation of tumor-relevant signaling pathways at the level of protein, we included signaling hallmarks from TGF-β/SMAD, NF-κB, JAK–STAT, PI3K/AKT/mTOR, and Wnt signaling pathways. The activation of these signaling effectors is often involved in cancer cell plasticity and tumorigenesis (Fig. 1B).

This workflow resulted in 894 942 high-quality single-cell proteomic profiles from 26 samples. To allow for a comprehensive view of TNBC "cytome", we generated two-dimensional maps from our data using the automated dimensionality reduction algorithm t-distributed stochastic neighbor embedding (tSNE). Most cells were of hematopoietic origin ($CD45^+$), followed by stromal ($CD90^+$) and cancer compartments ($PanCK^+$; Fig. 1C). Information about other cell types or cell subsets is not available due to the limited number of markers that the current stage of technology allows. The presence of major cell types was additionally confirmed by manual gating (Fig. S1A).

3.2. Ki-67-positivity and lymph node involvement at the time of surgery as a proxy for patient outcome

We expected that the population of cancer cells in our TNBC cases will be globally heterogeneous, with some



(B)

CANCER CELLS	MICROENVIRONMENT		SIGNALING & PROLIFERATION	
CD9	CD45	Pan-leukocyte marker	pNF-kB p65 (S529)	NF-kB activation
CD29	CD3	T cells	pStat3 (Y705)	JAK/STAT pathway
CD49c	CD56	NK cells, NK-T cells	pStat1 (Y701)	JAK/STAT pathway
ITGB5	CD14	Monocytes	pSmad2 (Ser465/Ser467)	TGFβ pathway
CD24	CD4	T helper cells	pSmad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad9 (Ser465/467)	TGFβ pathway
CD49f	CD8a	Cytotoxic T cells	pAkt (Ser473)	PI3K/Akt pathway
CD111	CD19	B cells	Non-p (Active) β-Catenin (Ser45)	Canonical Wnt
CD44	CD31	Endothelial cells	ROR1	Non-canonical Wnt
CD97	αSMA	Stromal cells	CD69	T cell early activation
CD112	Vimentin	Stromal cells	CD28	T cell costimulation
EpCAM	CD90	Stromal cells	CD38	Cell activation
PanCK			HLA-DR	MHC2 antigen presentation
			Ki-67	Proliferation

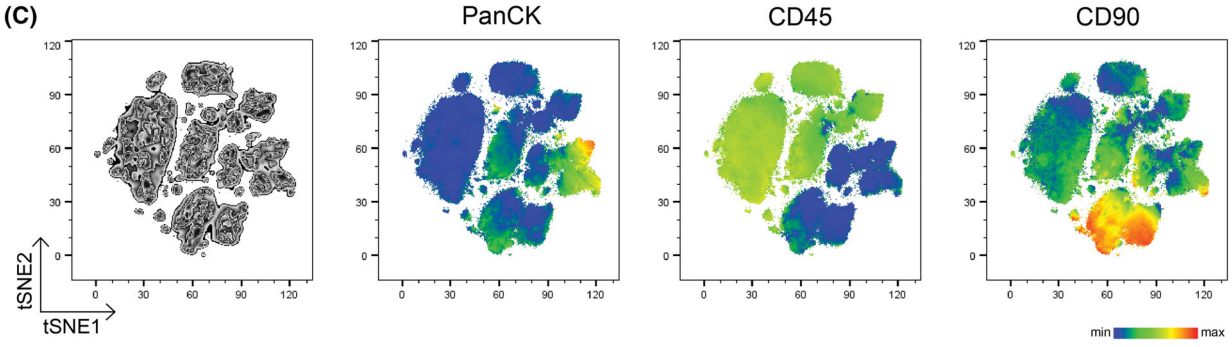


Fig. 1. Characterization of tumor heterogeneity in TNBC samples by mass cytometry. (A) Scheme depicting experimental approach and analytical workflow for primary TNBC patient samples used in this study. (B) List of cell surface and signaling molecules selected to characterize tumor and microenvironmental compartments. (C) Two-dimensional t-SNE visualization of PanCK, CD45, and CD90 expression in all cells and all samples (n = 26). The combination of these three markers was used for the identification of cancer (PanCK+), immune (CD45+), and stromal (PanCK-CD90+) cells, respectively.

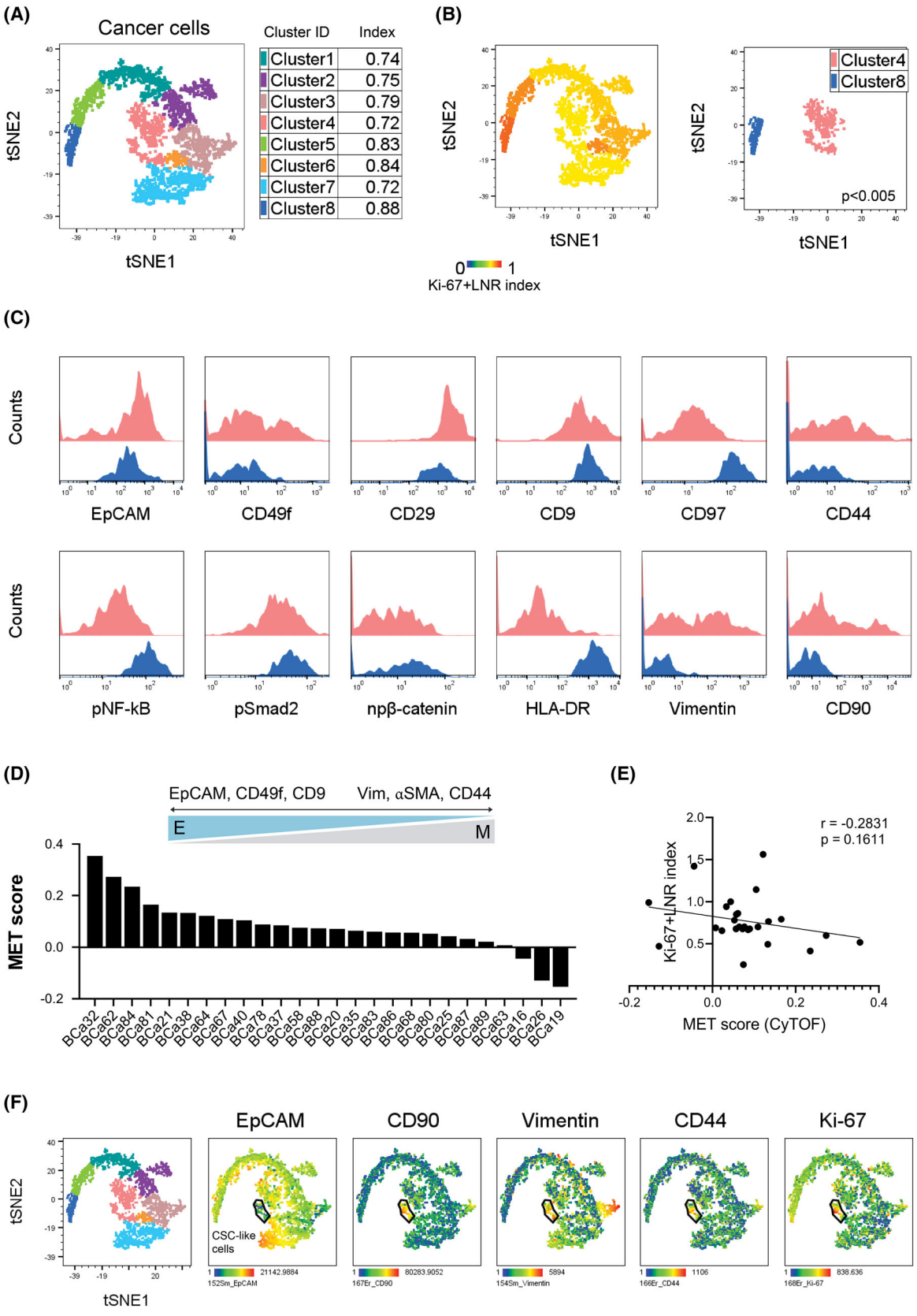
subpopulations more and some less abundant in aggressive tumors. To approach this question in an unbiased manner and to correlate cancer subpopulations with clinical parameters, we took advantage of a recently published automated and unsupervised machine learning algorithm RAPID [21]. This concept allows the identification of phenotypically distinct populations and determines whether they stratify patient survival. Due to the prospective design of this study that required freshly resected primary tumors, the long-term clinical outcome data, including metastatic spread and survival, was not available at the time of analysis. We therefore tested and robustly validated 'Ki-67 positivity and lymph node ratio' (LNR) at the time of the primary tumor surgery as a surrogate predictive and prognostic marker in TNBC, with minor modifications [31–33] (Fig. S1B). This approach enabled us to stratify patients solely according to their Ki-67% and lymph node involvement, as both parameters are routinely collected during diagnosis and widely available. As a proof-of-concept study, we correlated the introduced Ki-67 + LNR index with survival in two independent retrospective TNBC tissue microarray cohorts that we later used for histological validations (Fig. S1C,D). With good agreement between the predictive power of Ki-67 + LNR index and survival in retrospective cohorts, we applied this index to our prospective single-cell proteomic study.

3.3. Cancer subpopulations associated with clinical parameters

Simultaneous profiling of identically processed and preserved suspensions with mass cytometry eliminates the need for batch correction. We therefore applied modified algorithm employing FlowSOM clustering directly on cancer cells from our patients. FlowSOM provides an overview of how all markers are behaving on all cells, and detects subsets that might be otherwise missed. Using self-organizing maps for data analysis, FlowSOM serves as both clustering and visualization tool [21,24]. The identified FlowSOM clusters are then directly associated with Ki-67 + LNR index. Using this data analysis approach, we observed eight phenotypically distinct populations of $PanCK^+$ cells (Fig. 2A, Fig. S2A,B). Majority of analyzed tumors contained cells in all clusters (Fig. S2B, Table S2). These populations had distinct expression patterns of EMT/MET marker Vimentin, a number of cell adhesion molecules and integrins (CD29, CD49f, CD97, CD44, CD90), and signaling molecules (pNF-κB and HLA-DR; Fig. S2C,D). These TNBC subpopulations also differed in their Ki-67 + LNR indices. While Cluster 8 had the highest calculated Ki-67 + LNR index (blue; 0.88), Cluster 4 had the lowest (pink; 0.72) (Fig. 2B). Molecularly, cancer cells present in Cluster 8 displayed elevated expression of CD97, pNF-κB, and HLA-DR, and low CD90 and Vimentin levels (Fig. 2C). In contrast, Cluster 4 contained cells expressing low CD97, pNF-κB and HLA-DR levels, and high levels of CD49f, CD44, CD90, and Vimentin. The high expression of CD90 and Vimentin are frequently associated with epithelial-mesenchymal transition (EMT). EMT and its reverse mesenchymal-epithelial (MET) program are well-known contributors to plasticity and heterogeneity of cancers, including TNBC. We therefore decided to predict the EMT-MET phenotype of cancer cells in our patient cohort.

To assign cancer cells their EMT-MET status in an unbiased manner, we calculated the mesenchymal-to-epithelial (MET) score for each cell and all patients based on selected epithelial and mesenchymal molecules. We selected Vimentin, αSMA, and CD44 as *bona fide* mesenchymal markers, and EpCAM, CD49f, CD9 as the representative of epithelial phenotype (Fig. 2D, [30]). The score ranged from −3 value representing fully mesenchymal phenotype, to +3 for fully epithelial phenotype, with hybrid phenotypes scoring around 0. We assessed that the cancer cells in 23 of 26 samples are highly EpCAM-positive, with more than 50% cancer cells expressing this epithelial marker (average from all samples = 80.4%; 95% CI = 0.003) and exhibiting more epithelial phenotype based on their positive MET score. The MET score did not surpass 0.5 value, indicating that cells are, based on our classifier, in an intermediate or hybrid EMT/MET state, co-expressing both epithelial and mesenchymal proteins (Fig. 2D). MET score negatively correlated with Ki-

Fig. 2. Complex analysis of cancer cells in TNBC tumors. (A) t-SNE plot showing pooled cancer cells from 21 patients colored by FlowSOM clusters. (B) t-SNE plot colored with Ki-67 + LNR index (left), with depicted Ki-67 + LNR-high Cluster 8 (blue) and -low Cluster 4 (pink) populations in the right panel. (C) Histograms highlighting the difference in the expression of selected proteins in Ki-67 + LNR-high Cluster 8 (blue) and -low Cluster4 (pink). (D) Scheme depicting markers used for MET score computation (top) and plot showing calculated MET score for each patient (bottom). E, epithelial; M, mesenchymal. (E) Correlation of MET score with Ki-67 + LNR index in the mass cytometry TNBC cohort (r = Spearman correlation coefficient). (F) t-SNE plots highlighting identified CSC-like population colored by the expression of selected antigens. CSC, cancer stem cells.



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67 + LNR index (Fig. 2E), suggesting that cancer cells favor hybrid EMT/MET phenotype endowing them with high plasticity and increased fitness, features that are required for the tumor development and progression.

Quite unexpectedly, we also identified Cluster 4 to be enriched for small population of both EpCAM^{high} and EpCAM^{low} cells with CD90/CD44/Vimentin/Ki-67^{high} molecular profile, resembling proliferative cancer stem-like (CSC) subpopulation (Fig. 2F). Such basal-like CD44/CD90^{+} cells have been previously identified as tumorigenic and able to interact with monocytes and macrophages [34,35].

3.4. Unprecedented heterogeneity of TNBC stromal compartment

In parallel, using similar unsupervised analysis in the CD90^{+}/PanCK^{-}/CD45^{-} stromal fraction, we molecularly dissected 10 stromal subsets of comparable abundance, present in 24 tumor specimens (Fig. S3A,B). Mirroring the situation in cancer compartment, these stromal subpopulations displayed diverse phenotypes with the most significant differences in the expression of CD90, αSMA, and CD29 – a set of well-known cancer-associated fibroblast (CAF) markers, surface integrin CD49c, adhesion G protein-coupled receptor family member CD97, tetraspanin CD9, canonical TGF-β signaling hallmark pSmad2, and proliferation surrogate Ki-67 (Fig. S3C,D). Interestingly, these stromal clusters (Fig. 3A) showed similar Ki-67 + LNR index with only minor difference in values, with the highest being Cluster 4 (blue, 0.84), and the lowest detected in Cluster 9 (orange, 0.72; Fig. 3B). This observation suggested that the identified stromal subsets do not stratify patients based on Ki-67 + LNR index.

Compared to Cluster 9, cells in Cluster 4 (the highest Ki-67 + LNR index) expressed high levels of αSMA, CD90, CD29, CD49c, and Ki-67, also indicating an increased proliferative activity in this subpopulation (Fig. 3C). Because adhesion molecules and integrins, including CD49c, might play a functional role in tumor progression, we inspected whether these molecules are present also on stromal cells. We detected populations of CD49c-, CD49f-, and CD97-positive cells present across clusters at different proportions (Fig. 3D). All three surface molecules were concomitantly co-expressed, along with CD90 and CD44, with small "activated population" of cells expressing pSmad2, active b-catenin, and phosphorylated Akt (Fig. 3E). This suggests that these stromal subsets co-expressing signaling molecules might actively contribute to disease progression; however, precise functional experiments are needed. Although the stromal fraction did not differ in Ki-67 + LNR index, we observed heterogeneity in stromal compartment and identified interesting subpopulations for further studies.

3.5. Validation of complex TNBC phenotypes with routine, low-dimension immunohistochemistry

We next addressed the critical limitation of our study – the lack of long-term clinical follow-up in our freshly resected patient cohort – with an alternative strategy. We investigated the association between our mass cytometry protein hits with patient outcome in retrospective TNBC cohorts that included over 200 samples. We chose immunohistochemistry as a classical and routine procedure that does not require state-of-the-art instrumentation and is widely used at clinical pathology departments for diagnostic purposes.

First, we in-house assembled TNBC tissue microarray that included 108 primary tumors and if available, also matched lymph node metastases. For cross-validation purposes, this cohort included archival tissue from 24 patients analyzed with mass cytometry. This sample set was then clinically annotated for lymph node involvement and, if available, also for survival.

Based on the unbiased outcomes from mass cytometry analysis, we selected proteins which expression significantly separated the Ki-67 + LNR-high and -low clusters, in both cancer and stromal compartments. These included CD97, HLA-DR, pNF-κB, and αSMA. Additionally, we included known markers of EMT/MET that were included in the cytometric panel, specifically EpCAM, CD49f, and Vimentin (Fig. 4A).

Percentage of positivity and staining intensity of selected markers in stromal and tumor cells for individual patients was determined using the semiquantitative H-score method by a certified breast cancer pathologist in a blinded fashion. Although we were not able to reach statistical significance, this histopathological analysis revealed different expression patterns of EpCAM, CD49f, Vimentin, CD97, HLA-DR, and pNF-κB in lymph node metastases compared to matched primary tumors (Fig. 4B). These expression patterns varied from patient to patient and showed both increasing and decreasing trends in metastases relative to primary tumors, corroborating profound heterogeneity among the individual patients.

To approach this assessment in a similar way as done for the mass cytometry data, we classified the EMT/MET status of this retrospective histology cohort using histology MET score (hMET score). The

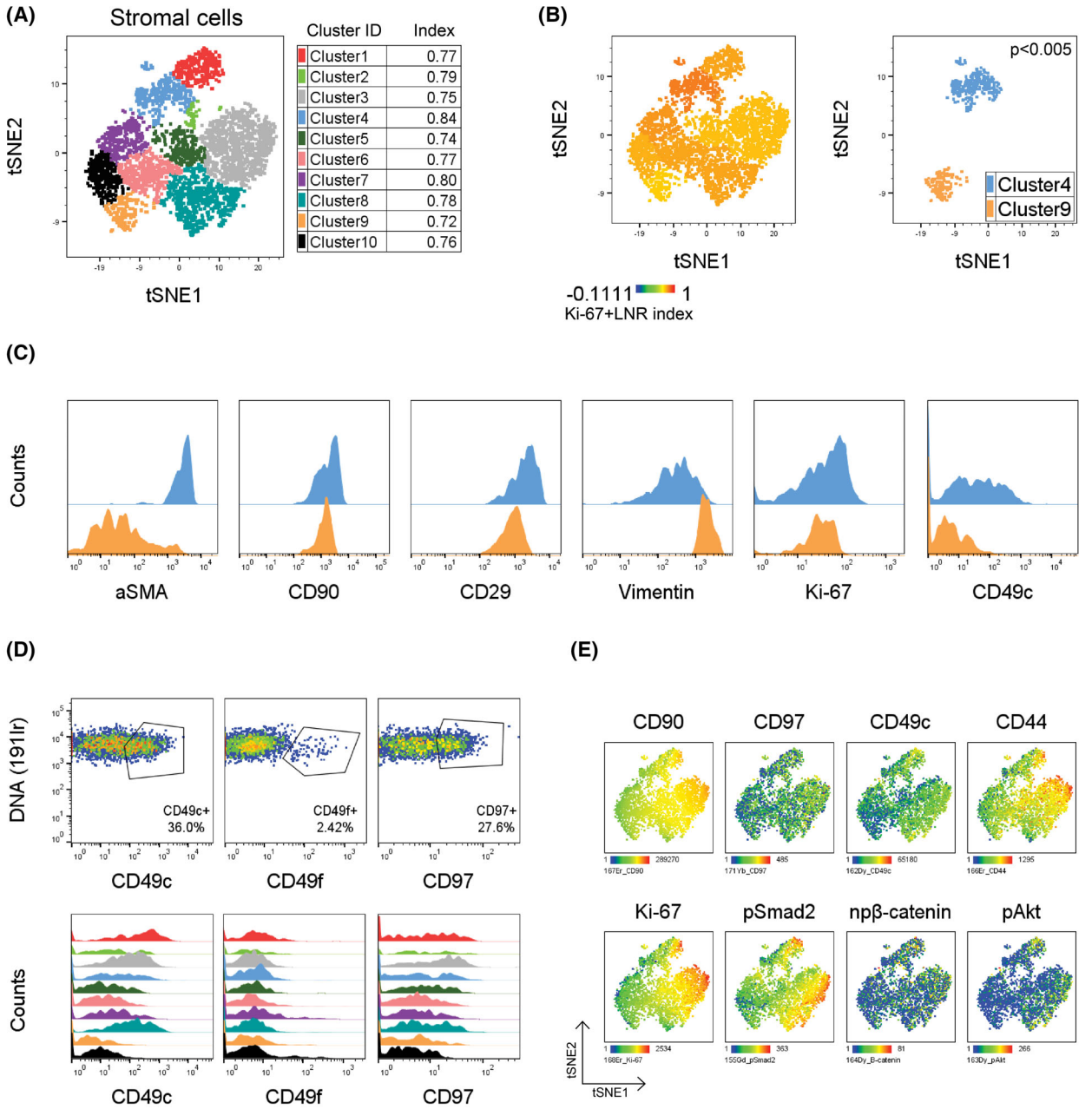

Fig. 3. Identification of TNBC stromal compartment. (A) t-SNE plot showing pooled stromal cells from 24 patients colored by FlowSOM clusters. (B) t-SNE plot colored with Ki-67 + LNR (left), with depicted Ki-67 + LNR-high Cluster 4 (blue) and -low Cluster 9 (orange) populations in right panel. (C) Histograms of selected stromal markers showing their expression in Cluster 4 (blue) and Cluster 9 (orange). (D) Subpopulations of CD49c-, CD49f-, and CD97-positive stromal cells from 24 patients, visualized as dot plots (top panel) and as histograms across all 10 stromal clusters (bottom panel). (E) t-SNE maps of stromal cells colored by expression profile of selected surface antigens and signaling molecules (npβ-catenin – active, non-phospho-beta catenin).

hMET score was calculated from H-score (0–300) of epithelial markers EpCAM and CD49f, and mesenchymal markers Vimentin and αSMA. Such hMET score extracted specifically from tumors that were analyzed with mass cytometry was in an agreement with the actual mass cytometry MET score (Fig. 4C). hMET score could be then, in simplified practice, used as a surrogate for mass cytometry MET score.

Out of all analyzed antigens, only the expression of EpCAM was significantly increased in tumors that

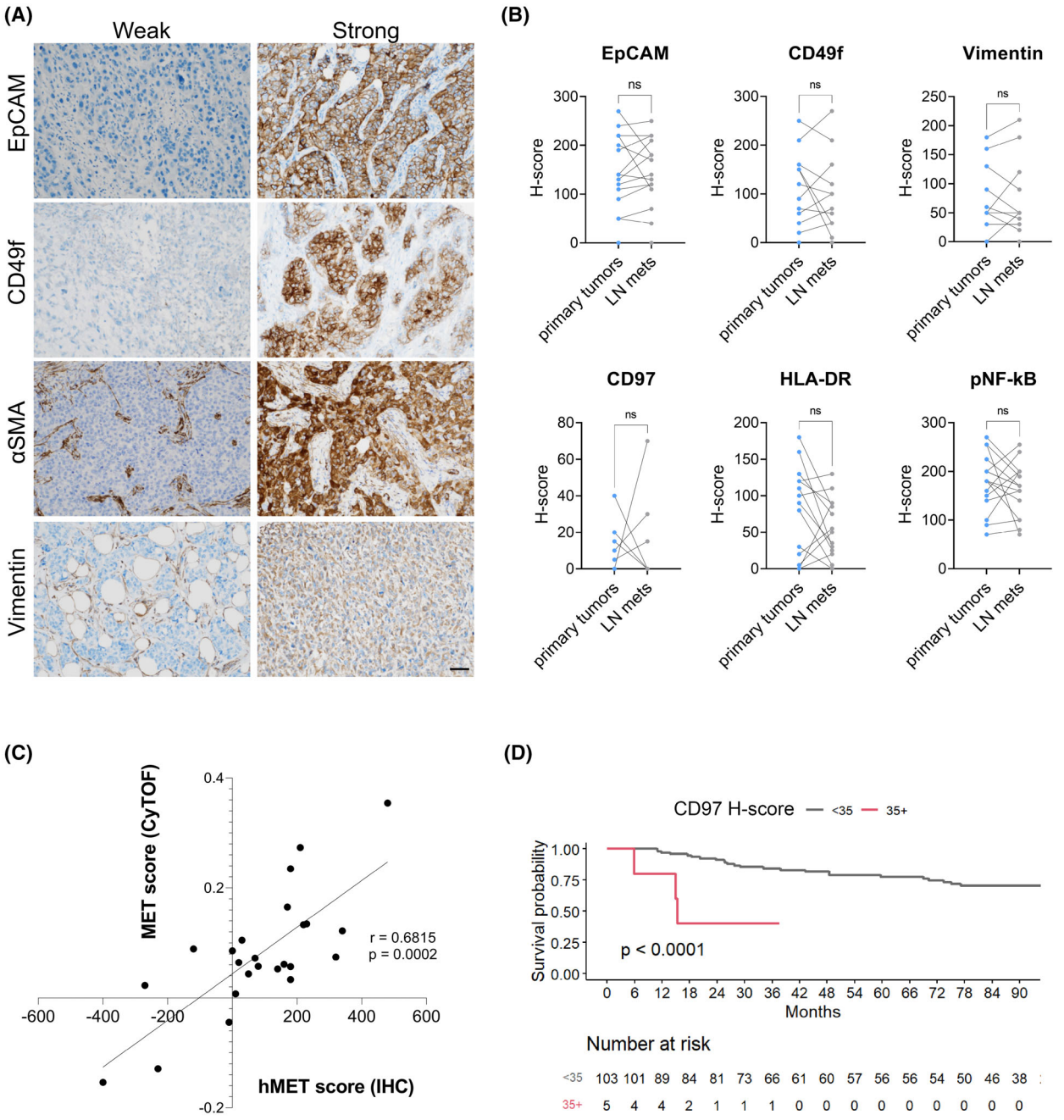

Fig. 4. Immunohistochemistry staining of molecules identified by mass cytometry in TNBC cohort. (A) Representative immunostaining showing weak and strong EpCAM, CD49f, αSMA, and Vimentin stainings in TNBC specimens, ranked based on H-score (paired *t*-test). Sample is representative from the cohort of 108 primary tumor patients. Original magnification 200×, scale bar – 100 μm. (B) Paired analysis of EpCAM, CD49f, Vimentin, CD97, HLA-DR, and pNF-κB staining in cancer cells in primary TNBC tumors and matched lymph node metastases ($n = 15$) evaluated by paired *t*-test. ns, not significant. (C) Concordance between mass cytometry MET score (CyTOF, *y*-axis) and histology MET score (IHC, *x*-axis) from the same cohort of 24 TNBC cases. r = Spearman correlation coefficient. (D) Kaplan–Meier curve showing overall survival of TNBC patients based on cancer cell CD97 expression. $n = 108$, logrank *P*.

disseminated into lymph nodes; the levels of EpCAM separated patients that had lymph nodes involved at the time of primary tumor resection from those that had not lymph node metastases present (Fig. S4A). Levels of HLA-DR and pNF-κB in the tumor fraction did not stratify patient survival (Fig. S4B). However,

increased cancer cell expression of CD97 was significantly associated with worse overall survival, in concordance with our mass cytometry data (Fig. 4D). Although the correlation of stromal Vimentin with survival did not show significance, we observed an interesting trend between elevated stromal αSMA expression and worse overall survival (Fig. S4C).

4. Discussion

The recent revolution in single-cell and spatial omics technologies revealed an unexpected level of TNBC heterogeneity. Despite this, the contribution of different tumor or stromal cell subsets to disease progression and therapy resistance remains unclear.

To understand how selected subpopulations and cell states shape tumor biology and influence the clinical outcome of TNBC patients, we established a single-cell proteomics pipeline, allowing complex analysis of TNBC heterogeneity with mass cytometry. This approach allowed us to characterize TNBC "cytome" and associated diverse cancer and microenvironmental phenotypes with clinical state of 26 patients at the time of tumor surgery. While most single-cell-based TNBC studies focus on immune cells [36] or solely rely on transcriptomic data [15,17,18,37], we used proteins as the ultimate readout.

Due to the prospective nature of our study, clinical outcome related to distant metastasis or patient survival was not available at the time of analysis. We therefore modified an alternative approach and introduced the Ki-67 + LNR index that reflects Ki-67% positivity and lymph node involvement at the time of surgery. Both of these parameters are relevant, standalone predictive and prognostic factors in TNBC, and their higher levels are associated with worse prognosis and more aggressive clinical features [31–33]. Nonetheless, there are some concerns about the reproducibility of the Ki-67 assessment and its limited clinical utility [38]. Recent Ki-67 consensus meeting established that Ki-67 IHC does have clinical validity for the determination of prognosis in patients with early-stage breast cancer and proposed several recommendations that can lead to precise analytical validity of Ki-67 IHC determination namely careful preanalytical handling and standardized visual scoring [39]. These methodical criteria were met in our study that involved early-stage TNBC patients (see Section 2).

Data analysis uncovered eight clinically distinct subsets of cancer cells, each with a specific protein signature. The cluster that significantly separated cell populations with high and low Ki-67 + LNR index was enriched in HLA-DR, pNF-κB, and CD97. CD97 also significantly stratified patients based on their overall survival in our histology studies. CD97 is a member of G-protein-coupled receptor family with involvement in adhesion, migration, and cancer progression [40,41]. CD97-positive cancer cells associated with higher Ki-67 + LNR index in patients and concomitantly expressed HLA-DR – an MHC class II molecule, normally expressed by the antigen-presenting cells, but also frequently identified in TNBC samples [42,43], and pNF-κB, a transcription factor implicated in TNBC proliferation and invasiveness with drug targeting potential [44,45]. Our findings suggest that the identified population highly expressing CD97/HLA-DR/pNF-κB proteins might be functionally involved in TNBC progression, and clinically targeted in the future.

In addition, we showed that the cancer cells in TNBC tumors reside in a spectrum of hybrid EMT/MET states based on the introduced MET score, consisting of key epithelial and mesenchymal markers. Such hybrid EMT/MET phenotype embodies a major survival advantage: it maintains a highly plastic and tumorigenic state that can be dynamically polarized toward EMT or MET, thereby promoting cancer cell spread or homing and metastatic outgrowth at distant sites [5,8,11,46,47]. In our study, the hybrid EMT/MET profile of cancer cells was further confirmed by an independent immunohistochemistry using the hMET score. This scoring in the matched samples was associated with high Ki-67 + LNR index and suggested that the hybrid phenotype may indeed drive tumor cell proliferation and metastatic lymph node colonization.

The stromal compartment of TNBC displayed a profound heterogeneity in cell surface and intracellular marker expression. We identified subsets of stromal cells co-expressing high levels of αSMA, CD90, and CD29 that associated with high Ki-67 + LNR index. This subpopulation resembled the profile of previously described myofibroblast-like ($\alpha SMA^{high}CD90^{high}FAP^{high}$) subsets of cancer-associated fibroblasts (myCAFs) [17,37,48]. These myCAFs are functionally capable to initiate EMT of cancer cells, hence support their spread to distant organs [49]. The TNBC stroma was also enriched for cell subsets positive for surface integrins CD49f and CD49c, and adhesion G-protein-coupled receptor member CD97. While the role of CAF-expressed integrins in breast cancer tumors has been previously suggested [50], their functional implications in the TNBC context remain unclear.

Our study comes with several specific limitations that are commonly associated with techniques

requiring tissue dissociation and more generally with mass cytometry-based approach. Firstly, although extensively optimized, the tissue dissociation protocol used in our laboratory may lead to underrepresentation of some cell types, including epithelial cells that are relatively fragile and can be destroyed during the dissociation process [51]. Secondly, this dissociation protocol can potentially alter the cell surface composition. Thirdly, pre-selected antibodies in this panel can bias the phenotyping attempts. For example, we considered the hereby identified stromal cells as cancer-associated fibroblasts, but we cannot exclude the possibility that $CD90^+$ fraction contained also mesenchymal stromal cells or perivascular-like cells. The part of the tumor tissue that was provided to us by the pathologists may not fully represent the composition of the entire tumor. Additionally, because of the prospective nature of our study, we lack information about clinical outcome (e.g. distant metastasis, overall survival) due to the short follow-up period.

5. Conclusions

In this study, we established a workflow for complex investigation of TNBC at a single-cell resolution and described phenotypically distinct cell subsets of tumor and stromal cells.

We introduced a novel clinically relevant Ki-67 + LNR index that can be associated with described heterogeneous cell subpopulations, thus identify cells that might contribute to disease development. Moreover, we identified cancer cell CD97 level as a predictor of worse clinical outcome. Taken together, our findings shed a new light on the heterogeneity of treatment-naïve TNBC, providing valuable resource for future research in both basic and translational settings.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

BK collected and processed patient samples, stained samples, analyzed and interpreted the data, and wrote and revised the manuscript. RF analyzed and interpreted the data. DK, AV, and TK performed mass cytometry experiments and helped with data analysis. JS helped with data analysis. PO performed statistical analyses in R software. JN managed clinical samples. PF, JB, and RO performed IHC and scored IHC TMA. JR interpreted the data and revised the manuscript. KS conceptualized and designed the study, interpreted the data, revised the manuscript, and supervised the study. All authors read and approved the final version of this manuscript.

Data accessibility

The data that support the findings of this study are available from the corresponding author [ksoucek@ibp.cz] upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Gating strategy of mass cytometry data and Ki-67+LNR index introduction. (A) Example of gating strategy showing the identification of live cells (CisPt-) and three populations of interest in a representative sample (BCa83): PanCK+ epithelial cells, CD45+ immune cells and CD90+ stromal cells. (B) Plot showing the calculated Ki-67+LNR index for each patient in TNBC cohort. (C) Kaplan-Meier plot showing the relationship between survival probability and high/low Ki-67+LNR index in a discovery cohort of archived TNBC patients that included samples used for mass cytometry measurement (n = 108). (D) Kaplan-Meier plot showing the relationship between survival probability and high/low Ki-67+LNR index in an independent, validation cohort of archived TNBC patients (n = 123).

Fig. S2. Unsupervised analysis of cancer cells in TNBC tumors. (A) t-SNE map of cancer cells illustrating identified 8 clusters associated with Ki-67+LNR index colored by FlowSOM clustering. (B) tSNE map colored by Ki-67+LNR index - left, Ki-67+LNR index values in all clusters on histogram - middle, contribution of cancer cells from patients (Sample ID) to identified clusters – right. (C) Histograms depicting expression of selected proteins in all cancer clusters. (D) Heatmap of normalized marker expression for different proteins in 8 clusters.

Fig. S3. Unsupervised analysis of TNBC stromal compartment. (A) t-SNE analysis of stromal cells illustrating identified 10 clusters associated with Ki-67+LNR index colored by FlowSOM clustering. (B) tSNE map colored by Ki-67+LN index - left, Ki-67+LNR index values in all clusters on histograms - middle, contribution of stromal cells from patients (Sample ID) to identified clusters - right. (C) Histograms depicting expression of selected proteins in all stromal clusters. (D) Heatmap of normalized marker expression of different surface and intracellular proteins in 10 clusters.

Fig. S4. Immunohistochemistry staining in TNBC TMA. (A) Expression of selected proteins in primary tumors with detected lymph node metastasis (LN met positive) versus primary tumors without lymph node metastasis (LN met negative; total n = 107). (B) Overall survival of TNBC patients from TMA cohort stratified based on NF-kB p65 and HLA-DR staining (H-score) in cancer cells. (C) Overall survival of TNBC patients from TMA cohort stratified based on αSMA and Vimentin expression (H-score) in stromal cells.

Table S1. Clinical and histopathological parameters of TNBC patients.

Table S2. Percentage of cells present in cancer clusters across samples.